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Cripto-1 (Cr-1) and Amphiregulin (Ar) are two EGF-like growth factors that occur at higher levels in breast cancer cells and tissues. We have shown that they occur in the late stages of mammary cell proliferation in virgin, pregnant and lactating mouse mammary glands. In order to test the roles of Cr-1, we infected CID9 normal mouse mammary cells with retroviruses over-expressing Cr-1, or with retroviruses that express an antisense version, in order to inhibit the production of this polypeptide. The result was striking: Cr-1 stimulated proliferation but inhibited differentiation (lactation). Reduction of Cr-1 decreased growth rate but the cells were able to differentiate almost as well as control cultures (control cells were infected with virus containing an empty vector). Similar studies on Ar are underway, and will be the focus of the next year.

We next sought to completely inactivate the Cr-1 gene in order to produce mice that expressed no Cr-1 in mammary glands as well as in all other tissues. To this end, we inactivated Cr-1 in embryo stem (ES) cells by homologous recombination and produced Cr-1 gene-targeted mice. In addition we targeted the Cr-1 gene in F9 embryonal carcinoma (EC) cells. Studies on these Cr-1(-/-) cells showed that ES cell differentiation to cardiac myocytes was prevented and EC proliferation was inhibited. Thus, Cr-1 is an oncodevelopmental gene, because it is a fetal product with proliferative and differentiative roles that is largely switched off in adult tissues but re-induced in cancer cells. We hoped to produce "knockout" animals with Cr-1 null mammary glands in order to determine if Cr-1 is important in the interaction between stroma and mammary epithelium during tumor formation or progression.

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E. D. Adamson 9/14/96

PI - Signature **Date**

TABLE OF CONTENTS

(5) INTRODUCTION

The distribution of Amphiregulin (Ar) and Cripto-1 (Cr-1) gene products as revealed by immunocytochemistry was thought to be restricted to tumors of the breast, ovary and a few other tissues, with little or no expression in adjacent normal tissues. However, there are some situations where Ar and Cr-1 expression do occur normally, and their activities and roles are of interest because we may start to understand the conditions that deregulate their expression or their activities. The epidermal growth factor (EGF) receptor is known to bind and respond to Ar, but Ar has a distinctive effect compared to the other 5 ligands for cells that respond to its signal. It is intriguing that Cr-1 is an orphan ligand, with no known receptor, since the truncation of the sequence within the EGF-like domain makes this ligand unable to bind to the EGF receptor. New information from the laboratory of Marc Kirschner, indicates that Cr-1 may be related to the fibroblast growth factor (FGF) family of ligands and may bind to an FGF receptor molecule but this has not yet been established. It has become clear that these genes that are EGF-like in sequence domains can stimulate the synthesis of other members of the family and can induce the expression of the epidermal growth factor receptor (EGFR). These events lead to autocrine stimulation of growth and to conditions that are conducive to genetic changes that may lead to cancer.

We have shown that Ar and Cr-1 are produced during the normal postnatal development of the mammary gland in mouse (Kenney et al, 1995). These growth factors are just perceptible among the proteins in the 12 week old mammary gland (using immunoblotting, immunocytochemistry and reverse transcription polymerase chain reaction, RT-PCR), but greatly increase during pregnancy and are at the highest level in late pregnant and early lactating glands.

This year, we have explored the roles of Cr-1 and Ar in mammary glands. We examined the roles of Cr-1 in mammary cells by over-expressing and by inhibiting Cr-1 expression in a normal mouse mammary cell line, CID 9 (this work has been submitted for publication). In order to completely negate the expression of Cr-1 in animals, it is necessary to target the gene with a gene disrupting vector using homologous recombination. We have made great strides in the long process towards the inactivation of the Cr-1 gene in embryo stem (ES) cells, in embryonal carcinoma (EC, F9) cells and in animals (see below).

We have also collaborated with Dr Lynn Wiley at the University of California at Davis (UCD) to show that Ar is produced by preimplantation embryos where it also appears to assist in the process of growth and development of the blastocyst (see below). Dr Wiley, her postdoctoral fellow and I wrote a review on the topic of EGF receptor activites in the preimlantation embryo for BioEssays

In summary, we have had to alter the tasks initially outlined partly because Dr Kenney left the lab and took some parts of the work with him in his new position at Georgetown University Cancer Center. Dr Kenney has attempted the over-expression of both Ar and Cr-1 in primary mammary epithelium cells, and the transplantation of these cells into syngeneic mammary fat pads. He has one paper published in December on the roles of amphiregulin in mammary development and cancer (Kenney et al, 1996) Cell Growth and Differentiation, 7, 1769-1781. We understand that another paper invovling Cripto-1 is in revision . In order to avoid repetition, we have followed essentially the same tasks using the same technique of mammary cell infection and transplantation, but using a normal mammary cell line instead of primary cell cultures. The two studies also differ in that the retrovirus that we use has a different promoter. The chief difference in the scope of the work is that Dr Kenney did not attempt to make *under-expressing* mammary cells because he did not make an antisense version of his vector, and so could not show that Ar has *autocrine* growth effects, but he has results that indicate that both Cr-1 and Ar have proliferative effects on primary mammary cells after transplantation. The Cr-1 work is not yet published and our knowledge of Dr Kenney's results is incomplete, since we no longer communicate.

The initial SOW was modified in order to respond to these changes in personnel, and to tackle the concept of gene inhibition with a more robust plan. The changes of plan were described in last years report. The initial aim to use a TGFα expression vector was in order to test the original retrovirus vector. This need was obviated by testing our retrovirus directly with Cr-1 expression. Other workers have produced significant data on TGFα effects in mammary cell growth that need not be repeated. Other changes in our detailed plans occurred in response to our own findings and the results of others . I assume that modifications to scientific experimental approaches are acceptable if they still aim to produce answers to the overall questions that were to be addressed.

6) BODY

i) Project to over- and under-express Cr in mammary cells.

The principal method to reveal the biological role of a gene product is to manipulate its expression in cells or in the whole animal. We can report significant progress in both these approaches. Dr Christine Niemeyer constructed a retroviral expression vector that can be used to infect mouse cells (CID 9 mammary cells) so that they over-express Cr-1 in one population, or have reduced Cr-1 expression in another. The control population was infected with an "empty" retrovirus. This is a necessary control to indicate if the infection produced an effect independently of the gene insert.

The results can be summarized as follows. Cripto was found to be expressed in CID 9 cells, a line of mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Our results showed that aberrant expression of Cripto affected the growth, morphology, and differentiation of these cells. Exogenous mouse Cripto expression from a retroviral vector caused CID 9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID 9 cells overexpressing Cripto did not differentiate efficiently. Infection of CID 9 cells with a Cripto antisense vector caused these cells to change in morphology, to grow slowly and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cripto is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the stimulation of proliferation at the expense of breast cell differentiation by Cripto. This result indicates a possible clinical approach for mammary tumor intervention. These results have been submitted for publication. When these Cr-1-under and over-expressing CID-9 cells were transplanted into syngeneic mice, they all produced mammary tumors, although, the literature indicated that the cells were, at one point, *not* tumorigenic. One conclusion was possible: the removal of expression of Cr-1 in CID9 mammary epithelial cells did not counteract the tumorigenicity of the tumors derived from these cells. Although not proven by this result, it appears that Cr-1 may not an initiator of the tumorigenic process. We hope to get further proof of this conclusion this year.

The disadvantage of using cell lines is that they change with increasing passage number. We were aware of this possibility and started with low passage cells specially obtained from Dr Mina Bissell. We have now obtained a similar mammary cell line, COMMA-D that is less tumorigenic and gave no tumor out of 5 transplants. The same retroviruses will be used to infect primary mammary epithelial cell cultures or COMMA-D cells and subsequently reconstitute mammary development by the transplantation of the cells into the mammary fat pads of syngeneic mice. We will hence determine if Cr-1 plays a role in normal mammary development and if the over-expression can stimulate the production of tumors. If autocrine stimulation and cross stimulation of the EGF-like genes occurs, it is possible that over-expression will lead to progression of proliferation to hyperplasia or carcinoma.

All the experimental approaches originally described, but modified by using COMMA-D cells, are in progress also for Ar, and are being performed by Dr Bradley Spencer-Dene. We have prepared Ar expressing retroviruses. Dr Spencer-Dene has also prepared a plasmid expression vector to over-express Ar using an inducible promoter, MMTV, which is idduced by dexamethasone. The additional feature that we are including is the addition of an epitope to a tag "FLAG", that can therefore be detected using a commercial anti-FLAG antibody. This allows us to assay the expression of the exogenous vector in cells that also express endogenous Ar. The antisense version of this has not yet been successfully made, after several attempts. We are planning a different strategy.

ii) Project to make antibodies to Cr-1 and Ar.

We have to report that we have had no success in raising antibodies to either Cr-1 or Ar. We had a peptide of 17 amino acids synthesized that represented the Pre EGF domain of Ar. Only the first bleed of the resulting rabbit antiserum gave some activity that was used to show the expression of Ar in preimplantation embryos. It turned out that the peptide was very impure and gave non-specific responses when used as an antigen. We are currently repeating this procedure with HPLC purified peptide and have made specially formulated immunogen as a mixture of 2 types of crosslinking with carrier protein. We have injected 2 rabbits and one chicken in order to obtain maximum antigenicity. The second bleed of the rabbit antibodies proved non-reactive and further booster injections are continuing. We have had no bleeds of the chicken yet. Meanwhile we have obtained anti human antibodies from Dr Gibbes Johnson, that work well enough on mouse tissues. Because this supply is limited, it is important that we obtain specific antibodies. These are necessary in

order to assay the level of expression of the Ar protein product in our studies to over- and under-express Ar in mammary cells.

We also made a bacterially synthesized fusion protein between the protein glutathione-S-transferase (GST) and Cr-1. The gel purified antigen proved not to be antigenic in 2 rabbits. We find that this was also the result obtained by Dr G. Persico, who is a collaborator providing the plasmid DNA for this study. Dr Persico failed to find antibodies in a total of 8 rabbits. We are continuing to use the antiserum raised by Dr Persico to a peptide within the mouse Cr-1 sequence. This gives a large number of high Mol. Wt bands on gels that we cannot account for, so this remains a significant problem. Expression studies can be performed by assaying the level of mRNA, but will not answer the question of whether the mRNA is translated and whether the role of the gene product is really being tested.

iii) Collaborative project with L.M. Wiley of UC. Davis. Dr Wiley's expertise is preimplantation development. My laboratory found that embryo stem (ES) cells, that are derived from blastocyst embryos , erxpress Ar. This meant that Ar could be expressed in preimplantation embryos. We provided the (transiently good) antibody that would detect Ar by immunocytochemistry, and the PCR oligonucleotides to Dr Wiley. Dr Tsark in Dr Wiley's lab showed that Ar is produced as early as the 8-cell stage of embryogenesis and is an autocrine factor that increases both rate of development /differentiation (time of onset of cavitation and trophoblast formation) and cell proliferation. It is significant that Ar influences the processes of growth and differentiation even in early embryo cells. It indicates the possibility that the stimulation of growth by Ar occurs not just in adult cells but also in cells that are immature or dedifferentiated, a state that describes tumor cells.

iv) Project to determine the time course of the expression of Cr-1 and Ar during tumorigenic progression.

We have examined the tumors produced in the transgenic mouse model (TGM) made by the insertion of the Polyoma middle T antigen behind the mouse mammary tumor virus (MMTV) LTR originated by Wm Muller. The females develop multifocal mammary tumors at around 20 to 34 days of age, while tumorigenesis in the males takes longer. Having found that both Cr-1 and Ar are highly expressed in the latter tumors, we are now doing a time course to determine if either of these proteins could act as markers for a specific stage of mammary tumorigenesis. We are following the expression of EGFR and ErbB2 during this time course because Ar can produce signals from both these receptor kinases.

We are planning a similar investigation on the transgenic mice (TGM) that over-express the neu (ErbB2) oncogene and the EGF ligand TGFα. These mice have been ordered from the Jackson Laboratory but are not yet available, and the study will be performed this coming year. We are also planning to investigate the TGM that we have prepared in another project in the lab, that under-express the epidermal growth factor receptor because of the expression of a dominant negative construct. The eventual aim is to make or obtain mice with different capabilities in mammary tumor formation and mammary cells with different production levels of of Ar and Cr-1. We will then investigate the effects of cross transplantation of mammary epithelial cells into the fat pads of tumoriogenic and normal mice to investigate the role of Cr-1 and Ar in the interaction of stroma with mammary epithelium. Making mice that expressed no Cr-1 in its mammary glands was part of this aim. The fall-back position for the over-expression of Ar in mammary glands is to make TGM that express MMTV-Ar. This will be indicated if the retrovirus construct does not work. We would like to eventually cross-breed all transgenic mice with tumorigenic potential in the mammary glands that are available, to determine the conditions and levels of Ar and Cr-1 expression that give rise to hyperplasia, dysplasia, carcinoma is situ and mammary carcinoma.

v. Progress report on the inactivation of Cr-1 in ES and F9 EC cells and in vivo.

Dr Chunhui Xu in the laboratory has made a targeting vector that recognizes the mouse Cr-1 gene. She has used the vector to perform the technique of homologous recombination in order to inactivate the Cr-1 gene in ES cells. There are 3 (or 4) parts to this study:- a) two of the ES cell clones that were assayed and found to contain a single inactivated Cr-1 gene were microinjected into mouse blastocysts (a pay back service at the Burnham Insititute) following published procedures for making "knockout " mice. We have 20 male chimeras and already have 2 litters of mice with proven germ-line inactivated Cr-1 in the heterozygous condition. We will next breed these mice and produce Cr-1 (-/-) mice without any Cr-1 expression. The analysis will depend on the viability of the offspring. We now expect that the homozygous embryo will die around day 7 to 9, due to the deficit in tissues that derive from at least a portion of the primitive streak (mesoderm). In particular, we expect that the heart will not develop properly (see below). As regards the mammary glands, we expect that the Cr-1

negative animal will not be viable and we will not be able to use the animals for the analysis of the ability of the Cr-1 negative mammary glands to be stimulated into the tumorigenic process as planned (see plan d).
b) the inactivation of the second Cr-1 allele in ES cells has produced cells with a major defect that is only obvious when the cells are cultured under conditions that lead to their differentiation. One of the most frequent types of cell produced by differentiated ES cells is the beating cardiac myocyte which are seen on the 7 th day and beyond. The ES-Cr(-/-) cells were unable to differentiate into cardiac myocytes. No beating was seen in 21 days of culture and no cardiac myosin protein was detected in immunoblots. This was specific because alphafetoprotein (AFP) was produced in normal amounts in all ES cell types, Cr-1(+/+), (+/-) and (-/-). AFP is another product secreted from differentiated ES cells. Moreover, the effect was dose dependent, because the (+/-) ES cells were 2 days delayed in their ability to give beating heart muscle.

c) Dr Xu has also targeted the Cr-1 genes in F9 embryonal carcinoma (EC) cells, because these cells (like ES) express high levels of Cr-1. In spite of the fact that these cells produce a number of other growth factors and do not respond to EGF family of proteins, they are clearly compromised in growth by the absence of Cr-1. Growth curves indicated that the F9 clones with a single or doubly inactivated Cr-1 gene grew less rapidly than the normal wildtype F9 cells. Therefore even in these cells that have one of the shortest doubling times of all cells (11 hours), Cr-1 plays a role in maintaining that rate of growth.

d) We have added a further experiment to obviate our possible inability to raise adult Cr-1(-/-) mice that can be used to examine the mammary glands. If there is enough time and funds in the fourth year, we will perform Cr-1 gene inactivation on tumorigenic and normal mouse mammary epithelium cells. The process of homologous recombination in somatic cells is not often attempted because the frequency of obtaining a targeted line is rare. However, we believe that we can do it because of the extraordinary high frequency of the event in ES cells where 70% of the clones isolated were found to be targeted. This will be our fall back position to make mammary cell lines lacking Cr-1 expression. We suspect that these cells will be less or unable to be rendered tumorigenic or will be protected from oncogenic conversions.

(7) CONCLUSIONS

Cripto-1.
1. Cr-1 protein contributes to the growth and development of normal mouse mammary epithelial cells. Cr-1 production is up-regulated by pregnancy hormones, while regressing mammary glands produce no Cr-1. The data indicate that Cr-1 plays a role in the events leading to differentiation (lactation) but **not** in differentiation itself. Reduction of Cr-1 levels inhibits growth and prevents the cells from reaching a stage with full differentiative capacity.
2. Data from homologous recombination studies (gene targeting) in ES and EC cells indicates that Cr-1 is needed to maintain the high rate of proliferation of undifferentiated EC cells.

3. Cr-1 plays a role in the events leading to cardiac cell differentiation at a stage before the synthesis of myosin.

Amphiregulin.
1. Ar is expressed in early mouse embryos as early as the 8-cell stage, in the inner cell mass and the trophoblast cells of the blastocyst. It plays a part in the development of the preimplantation embryo based on inhibition of cell numbers in embryos incubated in vitro with antisense Ar oligonucleotides.

2. Ar is expressed in the late stages of postnatal mammary gland development. It is produced maximally in pregnant glands and is less prominent in lactating glands. Ar is an autocrine growth factor for mammary cell proliferation.

(8) PUBLICATIONS

1. L. M. Wiley, E.D. Adamson and E. C. Tsark, (1995) Epidermal Growth Factor Receptor Function in Early Mammalian Development. BioEssays. 17, 839-846.

2. Niemeyer,C.C., Persico, M. G. and Adamson, E.D. 1996. Cripto is differentially expressed during mammary gland development and affects growth, differentiation and transforming potential (under revision for Cell Growth and Differentiation , Copy in the Appendix).

3. Tsark, E. Adamson, E.D. Withers, G.E. and Wiley, L.M. (1996) Expression and function of amphiregulin during murine preimplantation development. (under revision for Mol. Devel. Reprod.) (Copy in the Appendix)

Abstract:
Christina C. Niemeyer, M. Graziella Persico, and Eileen D. Adamson. (1996) Cripto is differentially expressed during mammary gland development and affects cell growth, morphology, transformation and differentiation. Abstract for a meeting in San Antonio in December, 1996. (Copy in Appendix).

Completed tasks for Cr-1

We have completed tasks 1-3 (omitting TGFα as explained earlier).
We have not attempted to do the time points indicated in task 4 because the cells were already rapidly tumorigenic, even without further expression of Cr-1.
We have completed task 5 for the current CID9 cells and will next use either primary or COMMA-D cells, which are less tumorigenic.
We have totally abrogated the activity of Cripto genes in ES and F9 E C cells. We now have Cr-1(+/-) mice.

Statement of Work

1. Tasks 1-3 for Ar will be completed using COMMA-D cells. This will test the effect of over- and under-expression of Ar in mammary epithelial cells and their ability to grow and differentiate normally.

2. The transgenic mice over-expressing polyoma middleT (MMTV-PyT) will be analyzed for Ar and Cr-1 levels during a time course to determine at which point Ar and Cr-1 might be important to the process of tumorigenesis.

3 Transplantation of mammary cells from MMTV-PyT mice into normal mice and the reverse will be performed (original tasks 6, 7 and 8).

4. We will also perform similar studies as described in task 2 on the TGM that we have prepared in this lab, that express a dominant negative mouse EGF receptor mutant transgene. These are to test the interaction of the stroma on the breast epithelium during mammary gland development and tumorigenesis.

5. Antibody production will remain a priority. We have much experience in antibody production, and will continue to use a number of approaches to increase antigenicity.

6. Analysis of the Cr-1 (-/-) mice during gestation, using cytohistochemistry to identify the cell types and tissues that are mutant.

(9) APPENDIX

CRIPTO, A PROTEIN HIGHLY CORRELATED WITH BREAST CANCER, AFFECTS CELL GROWTH, MORPHOLOGY, TRANSFORMATION POTENTIAL AND DIFFERENTIATION IN MAMMARY GLAND CELLS. Niemeyer CC*, Persico MG^, Adamson ED. The Burnham Institute, La Jolla Cancer Research Center, La Jolla, CA 92037, and ^International Institute Genetics and Biophysics, Naples, Italy.

The purpose of this study is determine the molecular characteristics of cripto during mammary gland development. The expression of Cripto, a member of the EGF-like family of ligands, is highly correlated with transformation in breast cancer. Eighty-two percent of breast carcinomas express Cripto whereas it is undetected in normal human breast tissue. We confirmed and extended findings that Cripto protein is expressed during the pregnancy and lactating stages of normal murine mammary glands but is barely detectable in glands from virgin animals and is undetectable in involuted glands. Cripto was found to be expressed in CID9 cells, a line of mouse mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Our results showed that aberrant expression of Cripto affected the growth, morphology, and differentiation of these cells. Exogenous Cripto expression from a retroviral vector caused CID9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID9 cells overexpressing Cripto did not differentiate efficiently. Infection of CID9 cells with a Cripto antisense vector caused these cells to change in morphology, to grow slowly and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cripto is an autocrine growth factor for normal breast cells, that when overexpressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the inhibition of breast cell differentiation by Cripto. The reduction of Cripto-1 expression in CID9 cells inhibited tumor formation in cells transferred to mammary fat pads. This indicates a possible clinical approach for mammary tumor intervention.

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FINAL
8/21/96

Cell Growth and Differentiation

Cripto is differentially expressed during mammary gland development and affects cell growth, morphology, transformation and differentiation.

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Running Title: Cripto: roles in mammary cell growth and development
Keywords: Retroviruses, overexpression, antisense, milk proteins, tumorigenicity.

Abstract

The expression of Cripto, a member of the EGF-like family of ligands, is highly correlated with transformation in breast cancer. Eighty-two percent of breast carcinomas express Cripto whereas it is undetected in normal human breast tissue. We confirmed and extended findings that Cripto protein is expressed during the pregnancy and lactating stages of normal murine mammary glands but is barely detectable in glands from virgin animals and is undetectable in involuted glands. Cripto was found to be expressed in CID 9 cells, a line of mammary epithelial cells derived from 14.5 day pregnant mice and we have used these cells to investigate the roles of this gene. Our results showed that aberrant expression of Cripto affected the growth, morphology, and differentiation of these cells. Exogenous mouse Cripto expression from a retroviral vector caused CID 9 cells to grow at an increased rate and to increased cell densities compared to parental and control cells. CID 9 cells overexpressing Cripto did not differentiate efficiently. Infection of CID 9 cells with a Cripto antisense vector caused these cells to change in morphology, to grow slowly and to achieve lower saturation densities but the cells were still capable of differentiating. We concluded that Cripto is an autocrine growth factor for normal breast cells, that when over-expressed stimulates excessive cell proliferation at the expense of the cell interactions that precede differentiation. The net effect is the inhibition of breast cell differentiation by Cripto. This indicates a possible clinical approach for mammary tumor intervention.

Introduction

Cripto (CR-1) belongs to the Epidermal Growth Factor (EGF)-like family of ligands that includes transforming growth factor-alpha (TGFα), heregulin (HRG), and amphiregulin (AR). The ligands differ in tissue of origin, binding affinities and mitogenic activities, but all are candidates for autocrine, juxtacrine, or paracrine effects in vivo. This family of proteins contain "EGF-like domains" with a highly conserved structure of three disulfide loops. Cripto has the 6 conserved cysteines that make up the EGF motif but the A-loop is deleted and the B-loop is truncated. At the carboxyterminus, an additional six-cysteine motif is present and conserved also in an analogous protein recently isolated from *Xenopus laevis*. (1). Thus, Cripto may be a ligand of another member of the ErbB (EGF) family of receptors or it could interact with a cofactor to bind to EGF receptor. It has been proposed that for *Xenopus*, Cripto may interact with an FGF receptor (1). Although there is no hydrophobic domain in the human gene product, the mouse protein has a signal sequence and is secreted (2-4). It has been proposed that the EGF-like sequences function as protein-protein contact motifs required for transfer of information.

No precise function has been attributed to Cripto but CR-1 protein is highly correlated with tumorigenicity. Of 68 biopsies on breast carcinomas, 82% expressed Cripto. Cripto expression has not been detected in normal human breast tissue or cell lines (5). Growth of two human breast carcinoma cell lines and the nontransformed human epithelial cell line 184A1N4 was stimulated by the addition of synthetic refolded human CR-1 peptides containing the EGF-like domain (2). All the human mammary tumor cell lines examined were found to express CR-1 using RT-PCR, Northern blot analysis and immunocytochemistry (3). The human CR-1 cDNA has also been overexpressed in mouse NIH3T3 cells and was shown to transform these cells such that they grew in soft agar in contrast to control cells (6). The same construct transformed an immortal mouse mammary cell line, NOG8 (6). Mammary tumors formed in transgenic mice overexpressing oncogenes TGF-α, neu, int-3, polyomavirus middle T antigen or simian virus 40 large T

antigen, all express Cripto-1 (7). In addition, Cr-1 expression was observed in pregnant and lactating mouse mammary glands (8). The latter observation suggested that Cripto played a role in differentiation as well as proliferation.

CR-1 was cloned as a full length isolate from a cDNA library derived from the human teratocarcinoma cell line NTERA2 clone D1. The TDGF-1 (CR-1) gene and an intronless sequence CR-3 were isolated and mapped on human chromosomes 3 and X, respectively (9, 10). CR-1 codes for a protein of 188 amino acid residues termed Cripto or *TDGF1* for teratocarcinoma derived growth factor-1(6). One murine gene, *Tdgf1* or Cr-1, and two intronless pseudogenes, Cr-2 and Cr-3 or *Tdgf2* and *Tdgf3*, have been isolated and characterized (4, 11). Cr-1 codes for a protein of 171 amino acids with 93% similarity to its human counterpart in the EGF-like domain. The molecular mass of Cripto protein varies according to the species and cell type: in human GEO colon and NTERA2/D1 embryonal carcinoma cells a polypeptide of 36 kDa predominates and it can be differently processed by glycosylation or modified in other ways. In mouse F9 cells only the secreted protein has been analyzed and a single species at 24 kDa was noted (2).

In the present study, CID 9 cells were used to examine the expression of Cripto-1 and to determine the effects of over and underexpression of this growth factor during growth versus differentiation. CID 9 cells are a subpopulation (12) of the COMMA-1D mammary epithelial cell line which was established from normal 14.5 day pregnant Balb/c mouse mammary gland tissue. They retain important characteristics of normal morphogenesis in vivo and functional differentiation in vitro (13). Normal CID 9 cells differentiate into alveolar-like structures (mammospheres) that express β-casein when grown on a laminin-rich extracellular matrix in the absence of fetal calf serum (FCS) and in the presence of lactogenic hormones (12).

We show here that Cripto is differentially expressed during mammary gland development and is expressed in a matrix dependent fashion in the normal mammary epithelial cell line. Inhibition of Cripto expression caused a change in cell morphology,

decreased cell growth, and reduced anchorage independent growth. However, the inherent tumorigenicity of CID 9 cells was not reduced. Mouse Cripto overexpression stimulated cell growth and decreased the differentiation potential of the mammary cells.

Results

Cripto is a pregnancy and lactation stage specific protein

Cripto protein, of sizes 24 and 26 kDa, is strongly expressed in the second phase of mammary gland development, pregnancy (Fig. 1, lane 2). This implies that its expression is driven by pregnancy-associated hormones, since involution of mammary gland tissue after pregnancy is associated with loss of Cripto expression (Figure 1, lane 4). In contrast, the virgin mouse mammary gland expresses extremely low levels (Fig. 1, lane 1). These results are in partial agreement with Qi et al.(5) in which no Cripto expression was seen in the virgin or post-involution stage of human mammary gland development. Cripto expression in pregnant mammary gland is supported by our observation that Cripto is expressed in CID 9 cells as a 24 kDa protein (Fig. 1, lane 7). When the cells differentiated on basement membrane substrates, they formed aggregate structures, started to express casein and more Cripto protein was produced. The protein was also modified to Mr 26 and 28 kDa (Fig. 1, lane 6). The CID 9 mammosphere structures appeared to be functionally similar to the in vivo pregnant-lactating mammary gland with stage-specific Cripto gene expression. This observation prompted us to test the hypothesis by experimental manipulation of Cripto expression in CID 9 cells.

Expression of exogenous Cripto.

In order to affect the expression of Cripto in mammary cells, a retroviral vector containing either sense or antisense Cripto was constructed. The polycistronic retroviral vector pGCEN (Fig 2) contains the encephalomyocarditis virus internal ribosome entry site (IRES) which allows efficient expression of multiple genes from a single proviral genome. Transcriptional controls and RNA processing steps that differentially affect expression of

the exogenous genes can be avoided (14). Cripto cDNA sequences (both sense and antisense) were inserted as described in the Materials & Methods section. Cripto cDNA and the selectable marker, neo^r are both expressed from a single promoter using the EMCV IRES insert. The pGCEN vector LTR is a promoter known to function in the mammary gland (15). CID 9 cells were infected with the retroviruses. More than 250 clones were selected in G418 and pooled so that the various cell phenotypes observed in these cells would be represented.

The level of Cripto protein expression in the infected cells was measured using Western blot analysis. Two different populations of CID 9 cells containing Cripto in the sense orientation were analyzed (Fig 3) and showed that Cripto was overexpressed at levels greater than two-fold higher than control vector populations (Fig 3). In the antisense populations only approximately one-fourth of the amount of Cripto was expressed compared to the controls (Fig 3). Thus, the retroviral vector constructs effectively modulated Cr-1 expression in CID 9 cells.

Morphology

Monolayer cultures of CID 9 cells commonly show two cellular morphologies, a spindle-shaped and a typical epithelial cobblestone pattern (Fig 4A). No differences were observed in the morphology of cells after infection with the empty vector compared to uninfected CID 9 cells (Fig 4, panels A and B). The cells infected with the sense Cripto cDNA also exhibited the same cellular morphology as the CID 9 cells or the pGCEN control CID 9 cells at both high and low cell densities. No significant morphological differences were observed during continuous propagation of these various pools. At low density, cells grew as islands of cells. In cultures remaining at confluence for 2 days, the presence of domelike structures was apparent. Cripto overexpressing cells formed more frequent and larger domelike structures (Fig 4C). Cells expressing antisense Cripto were different; the individual cells were flatter and spread to a larger area at both high and low

density. They showed the more typical cobblestone morphology of epithelial cells (Fig 4D) and at confluence, they did not form domes.

Cell growth analyses

To determine if over and underexpression of Cripto had an effect on mammary cell growth, various types of growth assays were performed. Cell growth rates were determined using maintenance culture medium. In this media, the CID 9 cells overexpressing Cripto (sense), underexpressing Cripto (antisense), and the control cells containing the empty vector (pGCEN) all grew at approximately the same rate for the first 24 hours. Then the cells overexpressing Cripto grew faster so that at 48 and 72 hours there were a greater number of cells compared to the control. The cells underexpressing Cripto appeared to reach confluence by 24 hours and the level of cells did not increase but decreased after 24 hours suggesting that they were starting to die. (Fig 5A).

To determine cell densities at confluence, cells were grown in 2% FCS, allowed to grow for 5 days and remain at confluence for 2 days and then counted (Fig 5B). The Cripto overexpressing cells grew to a greater density (3.2×10^5 cells/cm^2) than the control cells (2.4×10^5 cells/cm^2). For CID 9 cells expressing decreased levels of Cripto, the density at confluence remained at 1.2×10^5 cells/cm^2. Thus the cells containing the antisense vector showed contact inhibition at a statistically significant ($p < 0.05$) lower cell density compared to parental and control cells.

To determine if Cripto lowered the requirement of CID 9 cells for growth factors, cell proliferation assays were also performed by plating the cells in media containing 2% FCS to allow attachment and then growing them in serum-free conditions. Uninfected CID 9 cells and cells containing the sense or empty vector all grew at approximately the same rate and all the rates were lower than in 2% or 5% FCS conditions. The CID 9 cells containing the retrovirus with Cripto in the antisense orientation showed no growth in serum-free media and after several days these cells died (data not shown).

Colony formation and Anchorage independent growth

The Cripto-expressing sense CID 9 cells have a greater tendency to build up multilayers of cells in monolayer cultures compared to control pGCEN infected or uninfected CID 9 cells (Fig 6, A and B). The normal cells formed colony-like domes but Cripto underexpressing CID 9 cells on the other hand showed very little colony formation compared to control cells and ceased to proliferate after reaching confluence (Fig 6C).

The CID 9 populations showed differential abilities to grow in soft agar (Fig 6). Control cells formed 15 ± 3 colonies per dish (Fig 6D) whereas the Cripto overexpressing cells formed greater than 79 ± 6 colonies (Fig 6E). The antisense cells did not grow in soft agar and were therefore anchorage dependent for growth (Fig 6F).

Differentiation

Because the CID 9 cells differentiate on an extracellular matrix in the presence of lactogenic hormones, the various populations, containing either the sense, antisense, or empty pGCEN vector, were allowed to differentiate on matrigel. Morphological differentiation can be observed by the ability to form polarized epithelial structures termed mammospheres. The empty pGCEN infected CID 9 cells appeared the same as the CID 9 cells in that they formed frequent mammosphere structures (Fig 7A). The CID 9 cells overexpressing Cripto grew faster than the control cells and therefore there were more cells, however, no mammosphere structures formed. Interestingly, antisense-expressing cells also did not form any mammospheres but formed aster-like structures. This suggests that overexpression of Cripto in cells overrides the signals leading to differentiation that normally appear, while antisense expressing cells were too few to form mammospheres.

Biochemical indications for differentiation in mammary cells are the syntheses of milk proteins including β–casein. The CID 9 cells were grown in the presence of the lactogenic hormones, insulin, prolactin, and hydrocortisone either on an extracellular matrix or on plastic, and β-casein was detected by immunoblotting with an antibody to mouse milk proteins. Equal loading of gels was verified by immunoblotting with an

antibody to α-actinin (Fig 8, lower panel). The CID 9 cells overexpressing Cripto showed only about half the amount of β-casein expression as the control CID 9 population (Fig 8, lanes 2, 3, 11, and 12). This agrees with our morphological observations that Cr-1 overexpressing cells are predominantly proliferating compared to the control CID 9 or the normal CID 9 cells. Unexpectedly, the antisense containing CID 9 cells showed equal or greater expression of β–casein compared to the control suggesting a) that Cripto is not important for β-casein expression levels and b) that mammospheres are not important for differentiated expression. All populations of CID 9 cells (sense, antisense or empty vector) if grown in maintenance media without hydrocortisone and prolactin, either to confluence or not, failed to express β-casein (Fig 8, lanes 4-9).

A milk protein of approximately 22 kDa whose regulation was distinct from the control of β-casein expression (16) was produced by the CID 9 cells under various conditions of growth. Unlike β-casein expression, this protein was expressed in the cells whether lactogenic hormones were present or not. This protein was expressed at approximately the same level in all but one of the cell populations. The exception was the Cripto overexpressing CID 9 cells grown on matrigel in the presence of lactogenic hormones, which expressed only 15% of the level of expression of the 22 kDa protein observed with the other cells (Fig 8, lane 2). This property further distinguished the Cripto overexpressing cells.

CID 9 cells in vivo

To determine if the over or underexpression of Cripto had an effect on mammary gland development in vivo, we transferred CID 9 cells into the cleared fat pads of Balb/c mice. COMMA-1D cells, from which the CID 9 cells were derived, were previously shown to be non-tumorigenic in Balb/c mice (13). However, in our hands, parental CID 9 cells formed tumors in the transgenic fat pads of three out of three mice 5 to 6 weeks after transfer. In one mouse, a tumor of 15 mm diameter was examined. It contained no normal mammary cells or tissue and was invading the body cavity of the mouse. In a second

mouse, four distinct tumors were observed each 5 mm in diameter in the right #4 mammary gland in which the cells were placed. Control mammary glands that were sham operated remained normal. Thus, normal CID 9 cells are tumorigenic when transferred to normal mammary gland tissues in vivo. We also tested the tumorigenic potential of the antisense Cripto expressing CID 9 cells. They remained tumorigenic, giving rise to 6 out of 6 tumors 6 weeks after insertion into the mammary fat pad of syngeneic Balb/c mice. The tumor growth rates were similar and the cells were histologically indistinguishable. Western blot analysis showed that approximately one-fifth the amount of Cripto was expressed in the tumors formed from antisense Cripto CID 9 cells compared to the parental CID 9 cells (data not shown). Therefore loss of antisense effect could not explain the unchanged tumorigenic potential of the CID 9 cells in vivo.

Discussion

The mammary gland is a highly complex system of interacting cell types. It contains three epithelial compartments: luminal epithelium, alveolar epithelium, and myoepithelium (17). The mammary gland goes through four distinct postnatal developmental stages. Estrogen dependent ductal growth occurs from 6 to 8 weeks of age in the mouse when epithelial 'end buds' ramify from the nipple throughout the fatty mesenchyme creating a bush-like network of ducts. The spacing of the ducts and their morphology can be experimentally modified by the origin of the mesenchyme in which they are grown (18, 19), therefore mammary epithelial development is influenced by mesenchymal cells in the surrounding stroma. The onset of pregnancy initiates a second phase of development in which lobular-alveolar structures develop from the existing ductal system, in a process that is driven by pregnancy-induced hormones and estrogen (20). It was from this stage that CID 9 cells were derived. The lobular system grows and differentiates to form alveoli in which milk protein synthesis occurs during lactation, the third phase of development. Following weaning of the young, the mammary gland

undergoes extensive remodeling, leading to the loss of the alveolar structures, in a process involving large scale apoptosis (21). This last stage of development is termed involution. Each phase of mammary gland development requires a specific combination of systemic hormones that presumably activate different combinations of locally acting factors. Signaling molecules that have been implicated in local actions include members of the EGF, Wnt, FGF and TGF-β families (22-25). Each gene is expressed differentially during these developmental stages. We show here that Cripto is one of this group of genes, being strongly active during pregnancy and lactation and switched off during involution of the mammary gland after pregnancy. This suggests both that Cripto expression is regulated by pregnancy hormones and that Cripto has a function during these stages of mammary gland development.

In this study we took advantage of a mammary cell subpopulation that mimics pregnant mammary glands in vivo. CID 9 cells allowed us to assay both growth and differentiation events in culture. We found that Cripto was expressed at higher levels when cells were stimulated to differentiate by the presence of lactogenic hormones and extracellular matrix to lactogenic phenotypes, similar to pregnant glands in vivo. This strengthens the hypothesis that these hormones up regulate Cripto expression as they do amphiregulin (26) and TGFα (27, 28) in mammary tissue.

Although Kenney et al.(8) showed by RT-PCR that there is Cripto mRNA in the virgin mouse mammary gland, no 24 or 26 kDa proteins were observed. We were able to detect low amounts of a Cripto-protein of 26 kDa in virgin glands of 8 week and older mammary glands (Fig 1, lane 1). 24 kDa is the predicted and expected size of the mouse secreted Cripto protein demonstrated by Brandt et al. (2) to be authentic Cripto. In our analyses using immunoblotting of mammary gland tissues and cell lysates, we detected two Cripto proteins of 24 and 26 kDa (Fig 1) which are likely glycosylated or myristylated differently (2). Cripto protein is expressed in undifferentiated F9 cells as a protein of 24 kDa (Fig 1, lane 5) but this not produced by differentiated F9 cells (our unpublished data).

This is in agreement with previous experiments showing Cripto mRNA expression in undifferentiated but not in differentiated mouse F9 and human embryonal carcinoma cells (6).

How does the evidence presented here fit with the suggestion that Cripto is an oncogene? We show that aberrant expression of Cripto in breast cells affected multiple characteristics of the cells. Overexpression of Cripto caused cells to grow at a slightly higher rate in monolayer cultures compared to normal cells. Cripto also caused the cells to become less contact inhibited and allowed them to grow in aggregates and domes and also to grow anchorage independently (Fig 6). Together these results suggest that Cripto overexpression leads to a transformed phenotype. This would characterize Cripto as an oncogene. When Cripto expression is reduced in cells that normally express it, their morphology changes and they grow at one-third the rate of the control cells (Fig 5). This suggests that Cripto is an autocrine growth factor for CID 9 cells. Our data and the NOG-8 results (29) support the hypothesis that overexpression of Cripto leads to transformation and increased proliferation of normal mouse mammary epithelial cells. Our results also show that Cripto is required for normal growth and morphology of mammary cells during the pregnancy stage.

CID 9 cells differentiate in vitro and we were able to determine that Cripto has an inhibitory influence on differentiation. We showed that proliferation increased in CID 9 cells overexpressing Cripto while differentiation was inhibited as assessed by milk protein production (Fig 8). Two separate assays, mammosphere formation and milk protein expression, showed that overexpression of Cripto led to decreased differentiation. It is generally accepted that proliferation rates are inversely related to differentiation, and we demonstrated here that Cripto stimulated the proliferation needed to bring the cells to the state required (equivalent to pregnancy) for differentiation (lactation) but inhibited the differentiation stage.

We identified β-casein as a major product in differentiated CID 9 cells. Another milk protein of 22 kDa was also observed to be regulated independently of β-casein, a finding similar to that of Marte et al. (16) in HC11 cells which were also derived from COMMA-1D cells. Milk proteins are never observed in NOG-8 mammary epithelial cells or NIH3T3 fibroblasts (30). The 22 kDa milk protein appeared to be down regulated in CID 9 cells overexpressing Cripto when grown on an extracellular matrix. The nature of this protein and the mechanism of its regulation remains unknown.

One of our aims was to determine if Cripto is a transforming oncogene in mouse mammary tissue. CID 9 cells are an excellent model for mammary epithelial cell growth and differentiation in vitro, but they were also tumorigenic when transplanted into syngenic cleared fat pads. This was surprising since the original cells, COMMA-1D cells from which CID 9 were obtained (12), were shown to be nontumorigenic (13). The isolation and other procedures used to obtain CID 9 might have rendered them tumorigenic, or during the continuous passages they might have gradually become transformed. We showed here that some of the CID 9 cells (infected with empty vector) were able to grow in soft agar at the passage number used, suggesting they were already transformed. Thus, to determine if Cripto has a transforming effect on normal mammary gland development in vivo, primary cell cultures will have to be used and these experiments are in progress. It is intriguing, however, that the CID 9 cells express Cripto and form tumors in virgin mouse mammary glands whereas NOG-8 mouse mammary cells do not express Cripto and even when overexpressing Cripto do not form tumors in nude mice (29).

Some questions about Cripto need to be answered: What is the receptor for Cripto in CID 9 cells? What is the role of Cripto in oncogenesis? A ligand related to Cripto that activates the fibroblast growth factor receptor has been identified in *Xenopus* (1). The expression of Cripto is developmentally regulated in the mouse embryo (4, 31), but its role is unknown. Cripto first appears as a rare transcript in the blastocyst stage (day 4 postconception in mouse) in both the inner cell mass (ICM) and trophectoderm. Its

expression in mouse and human embryonal carcinoma cells derives from these early embryonic cells.

In summary, the results suggest that Cripto is positively regulated by lactogenic hormones and is important for mammary cell proliferation during the pregnancy stage of mammary gland development. Its function during pregnancy in the mammary gland may include the maintenance of correct cell morphology needed for lactation. Cripto also causes transformation and increases the rate of cell growth in mammary cells, a role suspected earlier because of its prevalent expression in breast and colon tumors. Using both in vitro and in vivo model systems we are addressing some of the questions concerning Cripto's regulation and role and more specifically its role in mammary gland development and cancer.

Material and Methods

Cell culture

CID 9 cells were kindly provided by Dr. Mina Bissell (Lawrence Berkeley Laboratory, Berkeley, CA). Cells were maintained in 1:1 DMEM:Hams F12 Nutrient Mixture (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FBS and insulin (5 μg/ml). For differentiation, the cells were grown for 7 days in the presence of lactogenic hormones (insulin 5μg/ml, hydrocortisone 1 μg/ml, and prolactin 3μg/ml) on Matrigel (Collaborative Biomedical Products, Bedford, MA). The cells were plated in 1:1 DMEM:F12 Nutrient mixture, 2% FCS, and lactogenic hormones at a density of 6×10^4 cells/cm^2. After 24 h the dishes were washed twice with PBS and fed with media containing no FCS but with lactogenic hormones.

Cell proliferation was quantified by determining formazan production from tetrazolium salt using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) and a ELISA plate reader programmed to quantify absorbance at 570 nm and background at 630 nm. All assays were performed under conditions where the 570 nm

absorbance readings were directly proportional to the number of cells/well. The anchorage independent growth assays were performed using a modification of method described by Stoker *et al.* (32). Cells were plated at a density of 4×10^4 cells per well of a 6 well plate in culture medium supplemented with 0.4% noble agar (Difco, Detroit, MI) over a lower layer of 0.3% agar, and allowed to grow for 21 days. Colonies were stained overnight with 0.05% p-iodonitrotetrazolium violet (Sigma Chemical Corp., St. Louis, MO), a vital stain that is taken up by mitochondria in cells.

Infections

The pGCEN vector was kindly provided by Dr. Richard Morgan (National Institutes of Health, Bethesda, MD) (Fig 2). The pGCEN neo retroviral vector expresses an inserted gene from the Moloney murine leukemia virus Long Terminal Repeat (LTR). An IRES sequence allows the expression of the neomycin resistance gene from the same promoter (33). The sense and antisense Cripto cDNA clones containing full length murine Cripto coding sequence was inserted into the XhoI site of the polylinker region in both sense and antisense orientations. PA317 producer cells (34) were transfected with 20 μg vector coprecipitated with calcium phosphate. Positive clones were selected with 800 μg/ml G418 after 5 days in culture. Supernatants free of G418 were collected and used immediately or aliquoted and stored at -70°C.

The CID 9 cells were infected with retroviral supernatant containing Cripto either in the sense or antisense orientation or the empty vector. The cells were plated at 8×10^5 cells per 100 cm^2 dish and the next day fed with 8 mls of viral supernatant (prefiltered through a 0.45 μm filter), 8 mls Hams F12 media, 4 μg/ml polybrene and 5 μg/ml insulin. After 24 h the infected cells were selected using G418 (400 μg/ml) in the media. Greater than 250 colonies were pooled and used in the studies as a mixed population. Because both Cripto (sense and antisense) and the selectable marker neo are expressed from a single

promoter using this vector, the cells were tested every few passages to determine that they maintained G418 resistance and hence Cripto expression.

Expression analysis

Mammary protein was obtained from mammary glands of staged mice. The tissues were homogenized in hypotonic buffer (20 mM HEPES, pH 7.4; 1 mM EDTA; 1 mM $MgCl_2$; 1 μg/ml phenylmethylsulfonyl fluoride; 20 μg/ml aprotinin) and solubilized in sample buffer (35). Culture dishes were washed twice in PBS and lysed in sample buffer. Cells grown on the extracellular matrix were first treated with dispase (Collaborative Biomedical Products) for one hour to dissolve the matrix and then washed and lysed as above. Equal amounts of protein were electrophoresed on a 15% SDS-PAGE gel and electrotransfered to Immobilon membranes (Millipore Corporation, Bedford, MA). Western blot analysis was performed and visualized using the ECL detection system (Amersham Corp., Little Chalfont, UK).

The rabbit polyclonal antibody was raised against a murine Cripto peptide, amino acid sequence 26 to 39, RDLAIRDNSIWDQK. The antimouse milk serum was a generous gift from Dr. N Hynes (Friedrich Miescher Institute, Basel Switzerland). This antiserum recognizes several milk protein including β-casein and the 22 kDa protein (16). Sheep anti-mouse casein antibody was kindly supplied by Dr. B. Vonderhaar (NCI). A rabbit polyclonal antibody to rat α–actinin was a gift from Dr. J. Singer (UC San Diego, CA) and served as a control for equal protein loading on the gel.

Mammary fat pad transplants

Mice were anesthetized with avertin. Mammary gland "clearing" was performed on the right inguinal #4 fat pads of mice at 3 to 4 weeks of age as described previously (36, 37). In essence, the nipple and primitive adjacent mammary epithelial tissue were excised while the remaining fat pad provided the region for growth of transplanted cells. The CID

9 cells were washed in serum-free medium and injected into the fat pads at approximately 1 x 10^5 cells per fat pad in 5 to 10 µl volumes (38). The skin was sutured, and mice maintained for 6 weeks to allow the transplanted cells to grow in the fat pad. Glands or tissues were dissected out, divided into portions and frozen for later analysis.

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Figure legends

Figure 1: Immunoblots of normal mammary gland tissue and cells to show Cripto protein expression. Cripto is highly expressed during the pregnancy stage (lane 2) and at a lower level at the lactating stage (lane 3) of mammary gland development. It is expressed at very low levels in virgin glands (lane 1) and is not seen in involuting glands (lane 4). Cripto is expressed as a 24 kDa protein in undifferentiated F9 cells (lane 5) and as both 26 and 28 kDa proteins in CID 9 cells grown on an extracellular matrix (Matrigel) for 7 days in the presence of lactogenic hormones (lane 6), compared to CID 9 cells grown in maintenance media on plastic (lane 7).

Figure 2: pGCEN retroviral expression vector used to over and under express Cripto in mammary cells. The Cripto cDNA was inserted in both orientations at the XhoI site (X). IRES, encephalomyocarditis virus internal ribosome entry site; LTR, Moloney murine leukemia virus Long Terminal Repeat sequences; neo, bacterial neomycin resistance gene.

Figure 3: Cripto expression levels in infected CID 9 cells. A) Overexpression of Cripto in CID 9 cells infected with the pGCEN-sense Cripto vector (sense) and underexpression in cells infected with the pGCEN-antisense Cripto vector (anti). α-Actinin was used to show relative protein levels in each lane. B) Graph of the percentage of Cripto expression ± SD in two different populations of infected cells compared to control pGCEN vector infected CID 9 cells (pGCEN) or uninfected CID 9 cells (CID 9) which was defined as 100%.

Figure 4: Morphological effect of Cripto over- and under-expression on CID 9 cells. Phase contrast micrographs compare the general appearance of the A) normal CID 9 cells with B) cells infected with control pGCEN vector, and C) cells infected with the Cripto expression

vector, and D) cells infected with the antisense Cripto expression vector. All the cells were seeded at the same density and allowed to grow the same length of time. For all panels the bar indicates 100 μm.

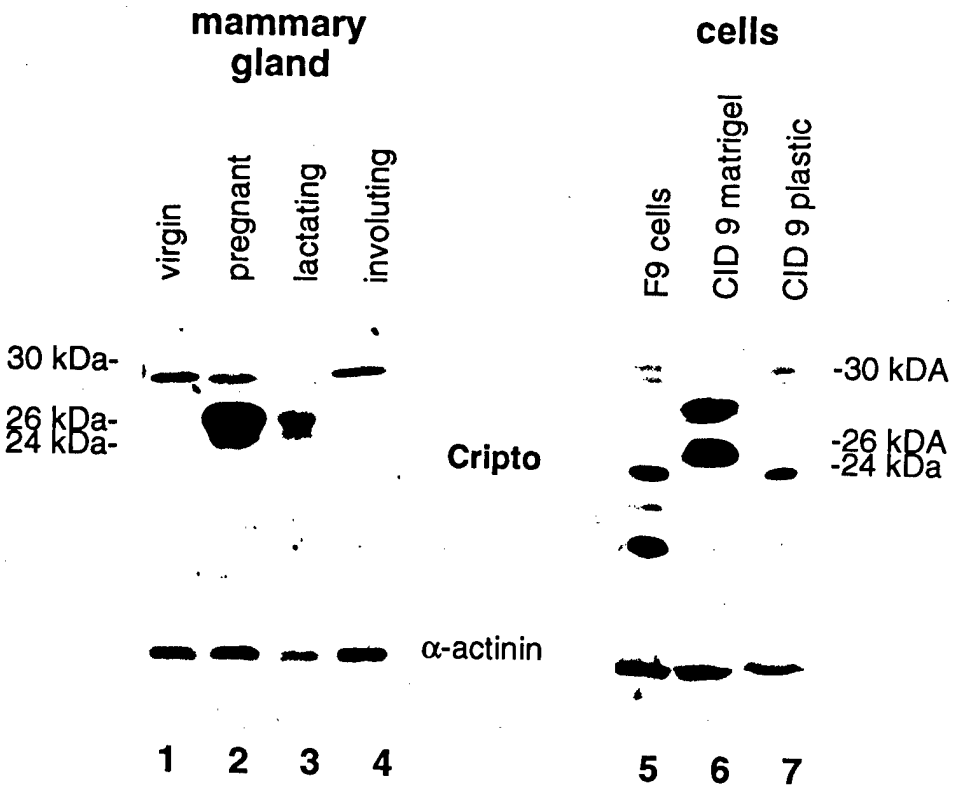
Figure 5: Cell growth assays. A) Growth rates of CID 9 cells containing the empty vector (pGCEN), the Cripto expression vector (sense), and the antisense Cripto expression vector (antisense). The absorbance at 570 nm is directly proportional to the number of cells/well. B) Monolayer density at confluence of cells expressing various levels of Cripto. Values are expressed as a percentage of the CID 9 cells containing the control pGCEN vector ± SD.

Figure 6: Colony formation in monolayer culture and anchorage independence (soft agar) assays of various populations. The CID 9 cells containing the empty vector (pGCEN) show some colonies formed both on plastic (A) and in soft agar (D). CID 9 cells overexpressing Cripto (sense) show a 3-fold greater number of colonies both on plastic (B) and in soft agar (E). Whereas, CID 9 cells underexpressing Cripto (antisense) shows very little or no colony formation on plastic (C) or in soft agar (F).

Figure 7: Morphological appearance of CID 9 cell populations grown on extracellular matrix (Matrigel) in the presence of lactogenic hormones. Phase contrast micrographs showing A) normal differentiated structures (mammospheres) formed in control population of CID 9 cells containing the empty pGCEN vector, B) overgrowth of CID 9 cells overexpressing Cripto, no mammosphere structures observed and C) aster-like structures formed when decreased levels of Cripto were expressed. All the cells were seeded at the same density and allowed to grow for 6 days.

Figure 8: Immunoblots to show proteins produced by populations of CID 9 cells grown under various conditions. CID 9 cells underexpressing Cripto (A), overexpressing Cripto

(S) or expressing normal levels of Cripto and containing the empty vector (P) were analyzed. Growth conditions included growth on Matrigel in the presence of lactogenic hormones for 6 days (Matrigel, lanes 1-3); growth on plastic in normal maintenance media until they were still subconfluent for 3 days (subc, lanes 4-6); growth on plastic in normal maintenance media until they were confluent for 5 days (conf, lanes 7-9); and growth on plastic in the presence of lactogenic hormones for 6 days (horm, lanes 10-12). The top panel indicates the Cripto expression in the populations. In the middle panel to compare the differentiation of cell populations, the 26 kDa β-casein and the 22 kDa milk proteins are indicated. α-Actinin staining was used as a control for equal loading of protein in the bottom panel.


mammary gland
cells
virgin
pregnant
lactating
involuting
F9 cells
CID 9 matrigel
CID 9 plastic
30 kDa-
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24 kDa-
-30 kDA
-26 kDA
-24 kDa
Cripto
α-actinin
1 2 3 4
5 6 7

Figure 1

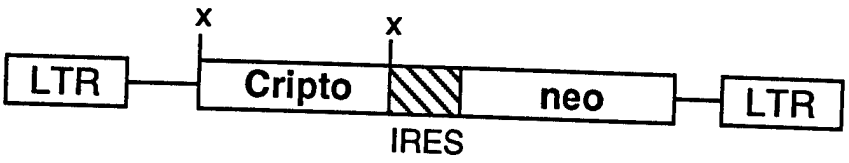

Figure 2

A.

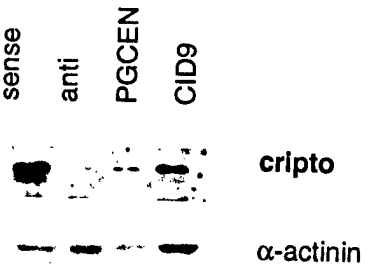

Figure 3A

B.

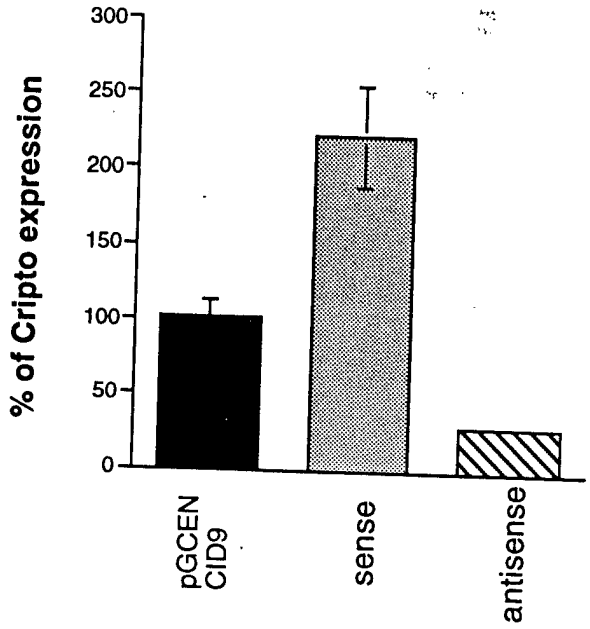

Figure 3B

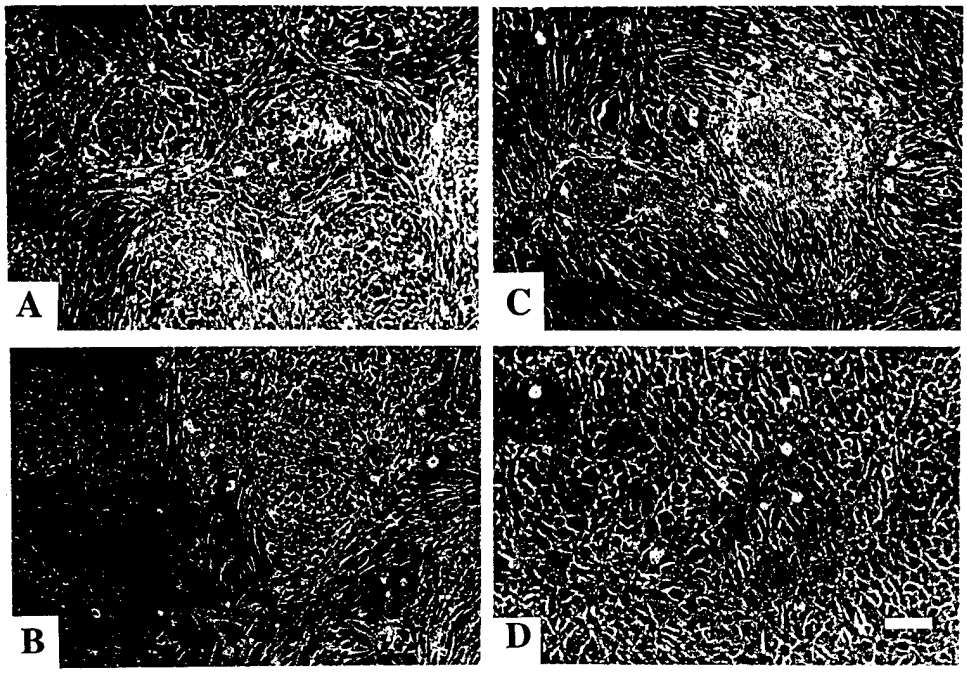

Figure 4

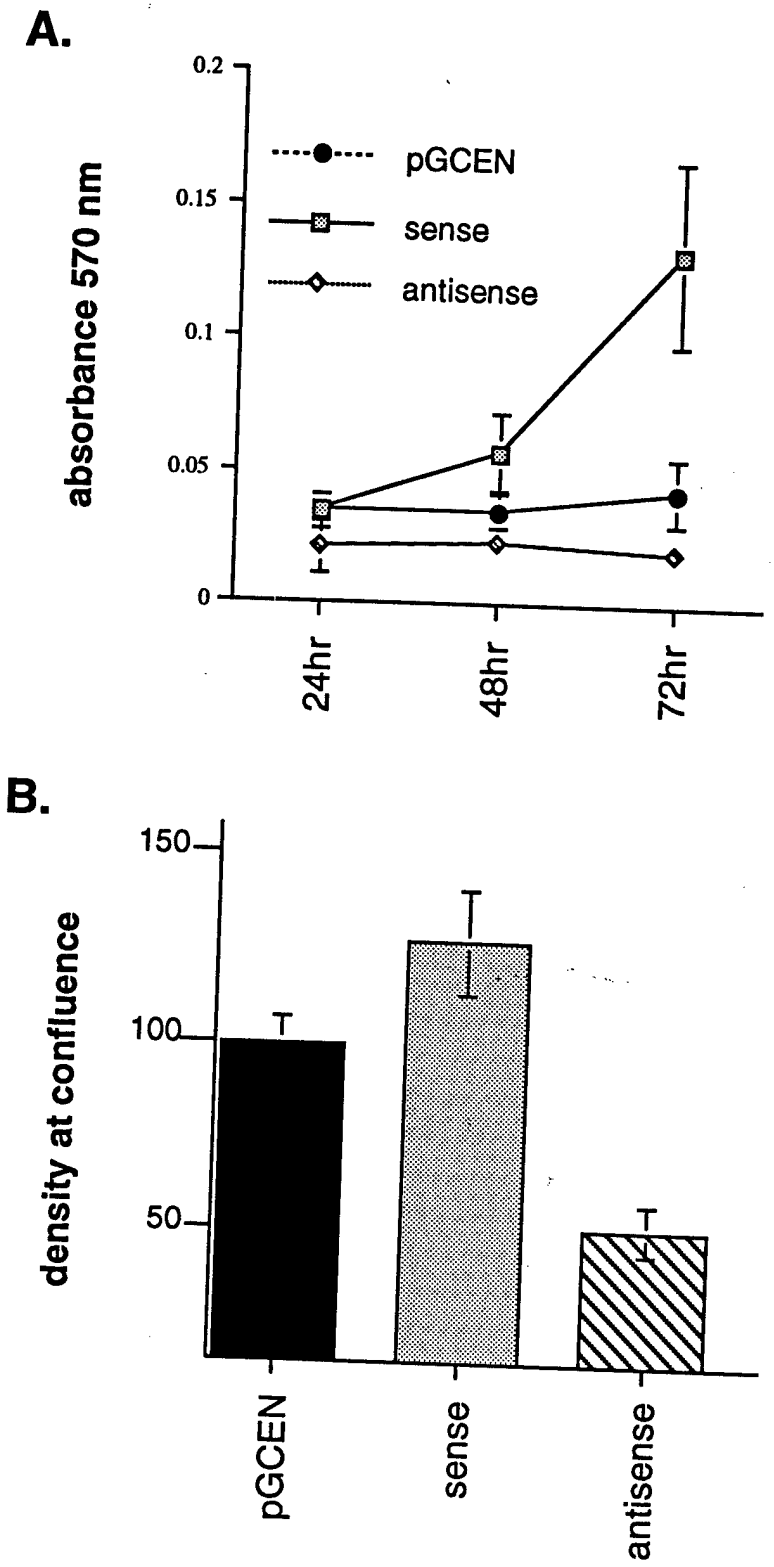
A.
absorbance 570 nm
0.2
0.15
0.1
0.05
0
pGCEN
sense
antisense
24hr
48hr
72hr
B.
density at confluence
150
100
50
pGCEN
sense
antisense

Figure 5

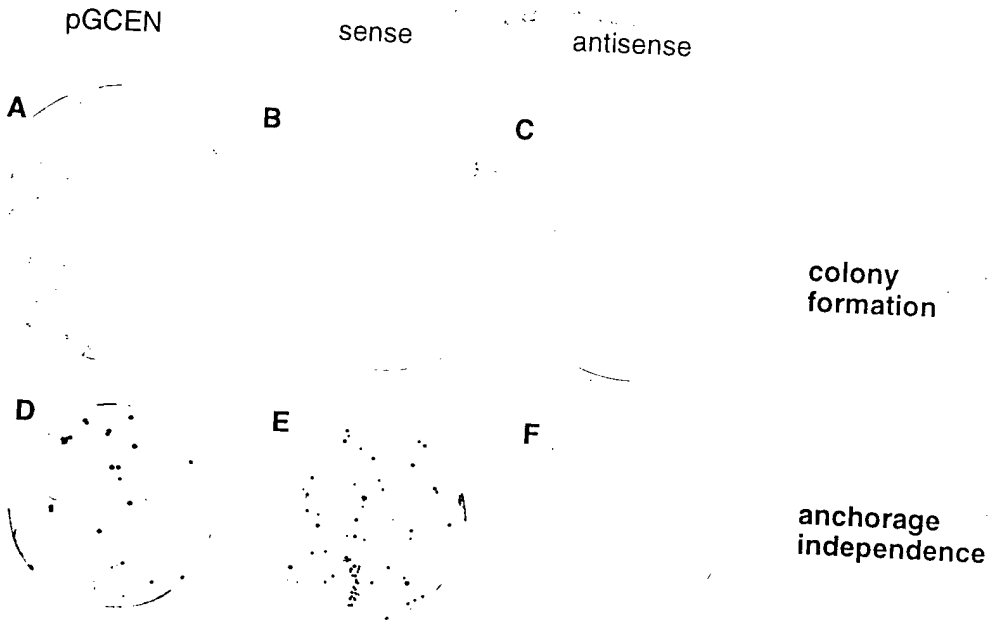
pGCEN
sense
antisense
A
B
C
colony formation
D
E
F
anchorage independence

Figure 6

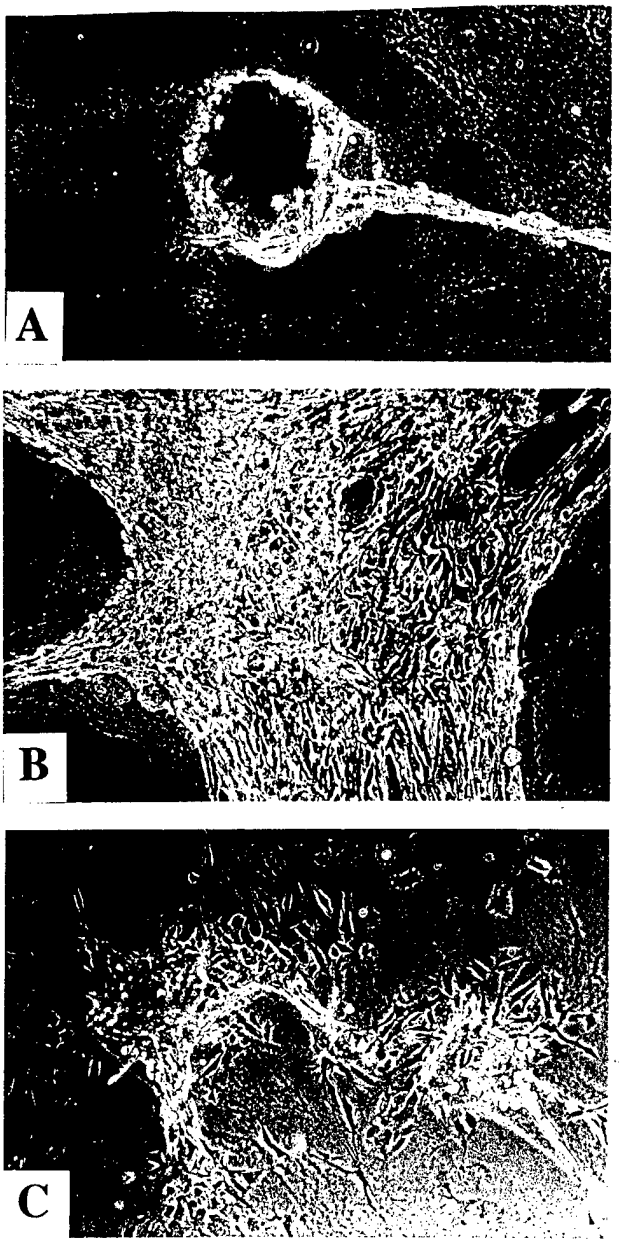

Figure 7

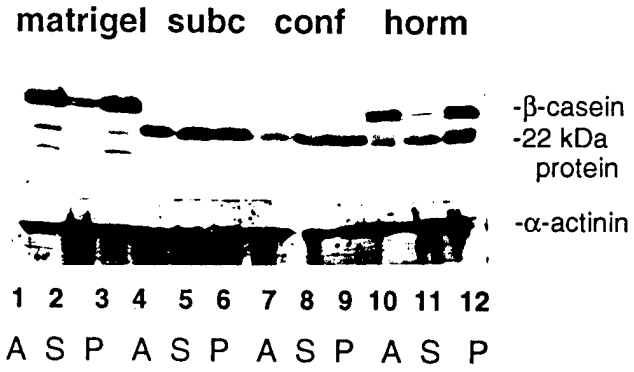
matrigel subc conf horm
-β-casein
-22 kDa protein
-α-actinin
1 2 3 4 5 6 7 8 9 10 11 12
A S P A S P A S P A S P

Figure 8

Expression and Function of Amphiregulin During Murine Preimplantation Development

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Abstract

Amphiregulin (Ar) is an EGF receptor ligand that functions to modulate the growth of both normal and malignant epithelial cells. We asked whether mouse preimplantation embryos express Ar and, if so, what the function of Ar is during preimplantation development. We used RT-PCR to show expression of Ar mRNA in mouse blastocysts and using a polyclonal anti-Ar antibody and indirect immunofluorescence, we detected the presence of Ar protein in morula- and blastocyst-stage embryos. Ar protein was present in both the cytoplasm and nucleus in both morulae- and blastocyst-stage embryos which is similar to Ar distribution in other cell types. **Embryos were cultured from the 4-cell stage or from the 8-cell stage in the presence of Ar to determine if Ar is functional during preimplantation development. Embryos cultured in Ar developed into blastocysts more quickly and also exhibited increased cell numbers compared to control embryos.** In addition, 4-cell stage embryos cultured in an antisense Ar phosphorothioate-modified oligodeoxynucleotide (S-oligo) for 48 exhibited slower rates of blastocyst formation and reduced embryo cell numbers compared to embryos exposed to a random, control S-oligo. TGF-α significantly improved blastocyst formation, but not cell numbers, for embryos cultured in the antisense Ar S-oligo. From these observations, we propose that Ar may function as an autocrine growth factor for mouse preimplantation embryos by promoting blatocyst formation and embryo cell number. We also propose that blastocyst formation is stimulated by Ar and TGF-α while Ar appears to exert a greater stimulatory effect on cell proliferation than does TGF-α in these embryos.

Key Words:

autocrine, blastocyst, RT-PCR, TGF-α

Introduction

The murine preimplantation embryo expresses several growth factor receptors and their ligands that are thought to account for the ability of the embryo to develop *in vitro* without added growth factors (for reviews see Wiley *et al.*, 1995; Schultz *et al.*, 1993; Heyner *et al.*, 1993; Adamson, 1993; Pampfer *et al.*, 1991). These embryo-produced growth factors and growth factor receptors appear to promote blastocyst development by stimulating both cell proliferation and development of the trophectoderm, a fluid-transporting epithelium responsible for blastocoele formation (Paria and Dey, 1990; Dardik and Schultz, 1991; Rappolee *et al.*, 1992; Brice *et al.*, 1993). More specifically, embryo cell proliferation seems to primarily be regulated by the IGF-I receptor and its ligand, IGF-II whereas trophectoderm differentiation seems to primarily be regulated by the EGF receptor and its ligand, TGF-α. (Dardik and Schultz, 1991; Rappolee *et al.*, 1992; Brice *et al.*, 1993).

Amphiregulin (Ar) differs from other EGF receptor ligands in that it contains two putative nuclear localization sequences, contained in a basic, hydrophilic NH_2-terminal region, which are believed to target Ar to the nucleus of several cell types (Shoyab *et al.*, 1989; Johnson *et al.*, 1991; Johnson *et al.*, 1992; Ebert *et al.*, 1994; Normanno *et al.*, 1994; Akagi *et al.*, 1995). However, Ar has many similarities with other EGF receptor ligands. For example, Ar binds to and induces autophosphorylation of the EGF receptor and p185 ^{erbB2} (Johnson *et al.*, 1993) and is proteolytically cleaved from a larger, transmembrane precursor that resembles a cell surface receptor (Plowman *et al.*, 1990). The sequence corresponding to the mature, soluble form of the growth factor is located in the extracellular domain of this precursor and its 40 amino acid COOH-terminal region contains 38% and 32% sequence homology to EGF and TGF-α, respectively. Ar also contains the six cysteine residues that interact to form three intramolecular disulfide bonds characteristic of EGF-like proteins.

In the present study, we asked two questions. First, do murine preimplantation embryos express Ar? Second, if embryos express Ar, what is the function of Ar during murine preimplantation development? In contrast to a previous report (Johnson *et al.*, 1995), our findings suggest that murine preimplantation embryos express both the mRNA and protein for AR and that it may be functioning as an autocrine growth factor by stimulating embryo cell proliferation and trophectoderm differentiation.

Materials and Methods

Embryos

Eight-to-twelve week old females of the CD-1 strain (Charles River, MI) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma) and then 48 hr later with 5 IU of human chorionic gonadotropin (hCG, Sigma) to induce superovulation and immediately paired with CD-1 males. The females were sacrificed 48 hr later by cervical dislocation and 4-cell embryos were flushed from the oviducts with modified Hank's basal salt solution (Goldstein *et al.*, 1975). The embryos were transferred to 20 µl drops of modified (Wiley *et al.*, 1986) T6 medium (Quinn and Whittingham *et al.*, 1982) overlaid with paraffin oil (Dow Chemical Co.) and placed in a water-jacketed incubator set at 37°C and filled with saturated 5% CO_2 in air.

Generation and Characterization of the Polyclonal Anti-AR Antibody

A peptide corresponding to residues 118 to 133 in mouse Ar with an added tyrosine residue to facilitate linking (Y-RKKKGGKNGKGRRNKK) was synthesized by the protein chemistry laboratory of the Burnham Institute. This peptide corresponds to a similar sequence used to make antibodies to human AR (Ab-2, Johnson *et al.*, 1993b) except that there were two amino acid differences between the human and mouse sequences. The peptide was linked to Keyhole limpet hemocyanin (KLH) protein to produce the immunogen. Five hundred µg peptide-linked KLH was homogenized with Freund's complete adjuvant for the first injection (subcutaneous) into 2 rabbits. Subsequently, 250 µg booster injections in Freund's incomplete adjuvant were made. The second to fifth bleeds were reactive to the peptide and to the protein in immunoblotting assays performed on pregnant mammary gland tissue lysates. Polypeptides of about 20 and 28 kDa were observed (data not shown) similar to published results (Kenney *et al.*, 1995, 1996). Immune IgG was affinity purified from antisera on peptide linked to Sepharose, eluted with

0.1 glycine-HCl, pH 2.5, immediately neutralized and dialyzed. Aliquots of affinity purified IgG were stored frozen at -70°C.

Indirect Immunofluorescence Assay for Detection of Ar Protein

Morulae and blastocysts were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and subsequently permeabilized with 0.05% Triton X-100 in PBS for 2 min. Triton X-100 has been shown to enhance the staining of Ar in the nucleus of cells (Akagi *et al.*, 1995; Johnson *et al.*, 1991). Fixed, permeabilized embryos were incubated in normal goat serum (undiluted) overnight at 4°C to block nonspecific binding sites and the zonae pellucidae were removed by brief exposure to acidic PBS (pH 2.5). Zona-free embryos were incubated for 30 min in the anti-Ar IgG at a concentration of 40 µg/ml. Specificity of the anti-Ar IgG was tested by preabsorbing the IgG with the immunizing peptide. Distribution of bound Ar IgG was visualized using rhodamine-conjugated goat anti-rabbit IgG (1:100) (Jackson ImmunoResearch Laboratories, Inc., PA). After rinsing with BSS/BSA, the embryos were transferred to a drop of BSS/BSA on a coverslip and immediately observed with an inverted phase contrast microscope fitted with epifluorescence illumination.

RT-PCR

Using the murine Ar cDNA sequence (Das *et al.*, 1995), two PCR primer pairs were designed. Primer pair #1 consisted of a forward (upper) primer with the sequence 5'-CAG GGG ACT ACG ACT ACT CAG A-3' and a reverse (lower) primer with the sequence 5'-GAT AAC GAT GCC GAT GCC AAT A-3'. Primer pair #2 consisted of a forward primer with the sequence 5'-AGT GCT GTT GCT GCT GGT CTT AG-3' and the same reverse primer as primer pair #1. Primer pair #1 gives a PCR product that is 434 bp in length and spans exons 2-4, whereas primer pair #2 gives a PCR product that is 613 bp in length and spans exons 1-4. The primer pairs were designed to span introns so we could distinguish

between products amplified from cDNA (reverse-transcribed from mRNA) and those amplified from contaminating genomic DNA.

The reverse primer was used for reverse transcription. In a thin-walled PCR reaction tube the following were placed: 100 mouse blastocysts transferred in approximately 2 µl of culture media, 2 µl of 5% NP-40, 5 µl sterile water, 2 µl reverse primer (0.5 µg/µl), 1 µl RNAsin (40 units/µl, Promega). This was then heated to 65 °C for 5 min., centrifuged quickly, then cooled to 4°C for another 5 min. To this mixture, the following was added: 5 µl of dNTPs (5mM each, Promega), 5 µl 5X MMLV-RT first strand buffer (Gibco BRL), 2.5 µl 0.1M DTT (Gibco BRL), and 1 µl MMLV-RT (200 units/µl, Gibco BRL). This was then incubated at 37°C for 1.5 h, heated to 80°C for 10 min., and then cooled to 4°C. The final volume yield of cDNA was approximately 25 µl. For a reverse transcription negative control (RT-), all the above reagents were added to another PCR tube except that 2 µl of sterile water was substituted for the 100 blastocysts.

PCR was then performed to detect cDNA made from Ar mRNA during the reverse transcription step. Primer pair #1 or #2 was used and in a thin-walled PCR tube the following were placed: 38 µl sterile water, 5 µl 10X Taq DNA polymerase buffer ($MgCl_2$ free, Promega), 3 µl 25 mM $MgCl_2$ (Promega), 2 µl dNTPs (10 mM each, Promega), 0.5 µl forward primer (0.1 µg/µl), 0.5 µl reverse primer (0.1 µg/µl), and 1 µl cDNA. For a PCR negative control (PCR-), all the above reagents were added to another PCR tube except that 1 µl of sterile water was substituted for cDNA.

Samples were heated to 95°C for 4 min, at which time 1.0 µl of Taq DNA polymerase was added for a "hot start" PCR. The samples were heated for 1 additional min at 95°C. Samples were then brought to the annealing temperature of 59°C for 30 sec and to an elongation temperature of 72°C for another 30 sec. The samples were then cycled 36 times through 93°C for 1 min, 59°C for 30 sec, and 72°C for 30 sec. The last cycle was held at 72°C for 10 min rather than 30 sec. The total number of PCR cycles was 37. PCR products were visualized on an ethidium bromide-stained 2% agarose gel under UV light.

Restriction Enzyme Digests of RT-PCR products

Primer pair #2 was used to generate a 613 bp product which was then subjected to restriction enzyme digestion. EcoRI was used as a negative control since the PCR product does not contain any EcoRI recognition sites. TaqI should cut the 613 bp product only once, producing restriction fragments 432 bp and 181 bp in length. Forty units of enzyme were used to digest 10 µl of PCR product in a 25 µl volume. Samples were incubated at 37°C overnight and heat killed the following morning. Products were visualized on an ethidium bromide-stained 2% agarose gel under UV light.

Antisense Ar Oligodeoxynucleotides

Phosphorothioate-modified antisense Ar oligodeoxynucleotides (S-oligos) were prepared as highly purified products by National Biosciences, Inc. Both the antisense and control S-oligo were capped with 4 phosphorothioate linkages at the 5' and 3' ends. The chimeric nature of these oligos have been shown to protect them against degradation by nucleases. The sequence of the antisense S-oligo was 5'-ACCGTTCACCAAAGTAATCT-3' which hybridizes to nucleotides 512 to 532 of murine Ar mRNA. The antisense S-oligo used is 100% similar to murine Ar mRNA and only 60% similar to other murine cDNA sequences reported in GenBank (Genetics Computer Group, 1992). The sequence of the antisense AR S-oligo was randomized to generate a control S-oligo. The sequence of the control S-oligo was 5'-TATATATATAAAGGCCCCCC-3' and is less than 65% similar to other known murine cDNA sequences reported in GenBank.

Embryo Culture in Antisense Ar S-oligos

Twenty- to -25 4-cell embryos were placed in 15 µl drops of modified T6 culture medium that contained either the antisense Ar S-oligo or the control S-oligo and was overlaid with paraffin oil. Concentrations of S-oligo were 25 and 50 µM. Additional

groups of 20-25 embryos were placed into 15 µL drops of culture medium lacking either the antisense or control S-oligo. The embryos were returned to the incubator and cultured for a total of 48 h. After the first 24 h, the embryos were transferred to new 15 µl drops of culture media containing fresh oligos and cultured for an additional 24 h. **At the end of the 48 h culture period, the embryos were scored for incidence of blastocyst formation and fixed to obtain mean embryo cell numbers using the method of Tarkowski (1966).**

Specificity Test for Antisense Ar S-oligos

Because isolated Ar was unavailable at the time of these experiments, TGF-α was used in an attempt to rescue embryos from the effects of the antisense Ar S-oligo. Four-cell embryos were cultured with or without TGF-α (10 ng/ml; Sigma) in 15 µl drops of culture media containing the antisense Ar S-oligo. Control embryos were cultured in 15 µl drops of culture media containing the random, control S-oligo. The S-oligos were used at a concentration of 50 µM which would be a more rigorous test of the ability of TGF-α to rescue embryos from the effects of the antisense Ar S-oligo. If the effects were entirely due to toxicity associated with the S-oligo, then these effects should not be altered by TGF-α. After a 48 h culture period, embryos were scored for onset of cavitation and fixed to obtain embryo cell numbers.

The ability of the antisense S-oligo to inhibit Ar protein production was also assessed using indirect immunofluorescence assays. However, these tests did not show a detectable difference in Ar protein expression between embryos cultured in the antisense S-oligo and embryos cultured in the control S-oligo. This failure may have resulted from a truncated, functionally defective Ar protein that retained its antigenic properties and thus was still recognized by the anti-Ar IgG. This possibility is consistent with the fact that this particular

antisense S-oligo hybridizes to the Ar mRNA at a position downstream of the nucleotide sequence that corresponds to the peptide used for immunization. Additionally, the precursor fragment of the Ar protein might be stabilized by its interaction with DNA.

Embryo Culture in Recombinant Amphiregulin

The 98 amino acid long form of recombinant Ar was purchased from R&D Systems. A stock solution was prepared by adding 1.0 ml of sterile PBS containing 2 mg of bovine serum albumin to 100 µg of lyophilized Ar. Five µl aliquots containing 0.5 µg Ar was prepared and 5 µl of a 2 mg/ml solution of bovine serum albumin was added to each 5 µl Ar aliquot giving a final stock solution consisting of 50 µg/ml Ar and 2 mg/ml BSA as a carrier protein. The Ar stock solutions were stored at -70°C. A sterile solution of 2 mg/ml BSA without Ar was also prepared as a control.

Approximately twenty 2-cell embryos were cultured in 20 µl drops of T6 culture medium containing Ar at concentrations of 1.0 ng/ml, 0.1 ng/ml or 0.01 ng/ml. Control embryos were cultured in 20 µl drops of T6 media containing an amount of BSA equivalent to what embryos cultured in Ar at a concentration of 1 ng/ml were exposed to. After 24 h in culture, embryos were placed into drops of culture media containing fresh Ar and returned to the incubator. Embryos were carefully monitored for the first signs of cavitation and scored for cavitation at 2 h time intervals. Embryos were fixed for cell numbers when at least 50% of control embryos had cavitated.

Eight-cell embryos were also cultured in the presence of Ar at concentrations of 10 ng/ml or 1.0 ng/ml. A higher concentration of Ar was included because of the shorter duration of exposure. Control embryos were cultured in T6 medium containing an amount of BSA equivalent to the amount present in media containing 10 ng/ml and 1.0 ng/ml Ar. Again,

embryos were monitored for the first signs of cavitation and scored for cavitation at 2 h time intervals. Embryos were fixed for cell numbers when at least 60% of control embryos had cavitated.

Statistical Analyses

Differences in mean embryo cell number were evaluated by unpaired one-way t-tests with a 5% significance level. Differences in percent of blastocysts were compared using chi square analyses with a 5% significance level.

Results

Localization of Ar Protein by Indirect Immunofluorescence

Control embryos exposed to the anti-Ar IgG which had first been preabsorbed with the peptide antigen did not exhibit any labeling, thus confirming the specificity of the antibody (Fig. 1b). However, morula- and blastocyst-stage embryos exposed to the anti-Ar antibody exhibited both a cytoplasmic and nuclear localization of fluorescence (Fig. 1c and Fig. 1d). Fig. 1c focuses on the nuclear signal produced in the morula and Fig. 1d focuses on the nuclear signal produced in the blastocysts. A nuclear distribution of Ar in preimplantation embryos is consistent with the nuclear localization of Ar reported for many other cell types (Johnson *et al.*, 1991; Johnson *et al.*, 1992; Ebert *et al.*, 1994; Normanno *et al.*, 1994; Akagi *et al.*, 1995).

RT-PCR and Enzyme Digests

To confirm the results obtained from the indirect immunofluorescence assays, RT-PCR was performed to determine if preimplantation embryos expressed the mRNA for Ar. Using primer pair #1, the predicted 434 bp product was detected in blastocysts (Fig. 2a, lane 2) whereas a band was not detected in the lanes corresponding to the PCR and RT negative controls (Fig. 2a, lanes 3 and 4).

To verify the RT-PCR product was amplified from cDNA reverse-transcribed from Ar mRNA, restriction endonucleases were used (Fig. 2b). Using primer pair #2, the predicted 613 bp product was detected in blastocysts. When the Ar RT-PCR product was treated with EcoRI, an enzyme that should not cut the RT-PCR product, the 613 bp product remained intact (Fig. 2b, lane 2). When the Ar RT-PCR product was treated with TaqI, the 613 bp product was cut into the expected 432 and 181 bp restriction fragments (Fig. 2b, lane 1).

Embryo Culture in Antisense Ar S-oligos

After 48 h of culture, embryos in the antisense Ar S-oligo lagged behind embryos cultured in the control S-oligo with respect to blastocyst formation and with respect to embryo cell numbers for concentrations of S-oligo, 25 and 50 μM (Fig. 3a and 3b; $p<.001$). In addition, embryos cultured in the control S-oligo at 25 and 50 μM concentrations exhibited significantly reduced mean embryo cell numbers compared to embryos cultured in T6 media lacking S-oligos (Fig. 3a and 3b; $p<.05$ for 25 μM and $p<.001$ for 50 μM). This served as an indicator of the degree of toxicity associated with the S-oligos used in this study. Since the antisense Ar S-oligo significantly reduced both the rate of blastocyst formation and mean embryo cell number compared to embryos cultured in the control S-oligo, this suggests that some of the effects produced by the antisense S-oligo may have resulted from a sequence-specific effect of the antisense S-oligo on Ar protein function rather than toxicity associated with the antisense S-oligo.

The morphology of the embryos cultured in the antisense Ar S-oligo was very different from the morphology of embryos cultured in either the control S-oligo or T6 media alone (Fig. 4). We consistently observed timepoints when 60-70% of embryos in control groups had formed blastocoeles while 100% of the embryos cultured in the antisense Ar S-oligo were developmentally delayed at the morula stage (Fig. 4).

Specificity Test for Ar S-oligos

TGF-α significantly improved the rate of blastocyst formation in embryos cultured in the antisense Ar S-oligo compared to embryos cultured in the antisense Ar S-oligo without TGF-α (Fig. 5; $p<.001$). However, the rate of blastocyst formation for these embryos was still significantly slower ($p<.05$) than for embryos cultured in the control S-oligo. In addition, embryo cell numbers were not significantly improved in embryos cultured in the antisense Ar S-oligo and TGF-α (data not shown). This suggests that TGF-α can not compensate completely for Ar function. The inability of TGF-α to rescue embryo cell

numbers was not surprising since we have found that addition of TGF-α to the culture medium of preimplantation embryos stimulates primarily blastocyst formation rather than embryo cell number (Fig. 6).The morphology of the embryos cultured in the antisense Ar S-oligo and TGF-α is shown in Fig. 7.

Embryo Culture in Amphiregulin

Two-cell embryos cultured in the presence of Ar at 1.0, 0.1 and 0.01 ng/ml developed into blastocysts significantly faster than control embryos (Fig. 8a, Fig. 8b). In addition, the mean embryo cell number for embryos cultured in Ar at 0.01 ng/ml was significantly greater than the mean embryo cell number for control embryos (Fig. 8c, $p < 0.02$). Embryos cultured in Ar at 1.0 and 0.1 ng/ml also had greater mean embryo cell numbers compared to controls, but this increase was not statistically significant.

Eight-cell embryos cultured in Ar at 10 and 1.0 ng/ml also developed into blastocysts more quickly than control embryos (Fig. 9a, Fig. 9b). Both concentrations of Ar produced significantly greater mean embryo cell numbers compared to controls (Fig. 9c).

Discussion

Here, we provide the first evidence that murine preimplantation embryos express the EGF receptor ligand amphiregulin (Ar) in addition to TGF-α (Rappolee *et al.*, 1988; Werb, 1990). RT-PCR was used to show expression of Ar mRNA in mouse blastocysts and an affinity-purified anti-Ar IgG was used to demonstrate a cytoplasmic and nuclear localization of Ar protein in morula- and blastocyst-stage embryos. Immunohistochemical analyses of several cell lines have shown Ar to be present in both the cytoplasm and nucleus (Shoyab *et al.*, 1989; Johnson *et al.*, 1991; Johnson *et al.*, 1992; Ebert *et al.*, 1994; Normanno *et al.*, 1994; Akagi *et al.*, 1995).

Ar was so named because of its dual roles in modulating growth: it can promote or inhibit the growth of both normal and malignant epithelial cells depending on the concentration of Ar the cell is exposed to (Shoyab *et al.*, 1988; Johnson *et al.*, 1991). Phosphorothioate-modified antisense Ar oligodeoxynucleotides reduce cell growth in a gastric carcinoma cell line and human mammary epithelial cells, suggesting that Ar acts as an autocrine growth factor for these cells (Normanno *et al.*, 1994; Akagi *et al.*, 1995). This negative effect on cell proliferation is similar to what we observed for embryos cultured in antisense Ar S-oligos (Fig. 3a and 3b). In the work presented here, we used an antisense Ar S-oligo that should prevent the synthesis of sequences corresponding to the third disulfide loop in mature AR and the transmembrane and cytoplasmic domains contained in the precursor. The six cysteines and their disulfide loops in EGF and TGF-α are essential for biological activity (Heath *et al.*, 1986). The transmembrane domain would be required for insertion of the AR precursor into the plasma membrane to provide the potential of cell membrane-bound Ar interacting with EGF receptors on adjacent cells. Stimulation of EGF receptors by membrane-anchored TGF-α is known to occur and has been termed juxtacrine stimulation (Brachmann *et al.*, 1989; Wong *et al.*, 1989; Anklesaria *et al.*, 1990). In our experiments, as well as in those using other cell types, it is possible for the antisense Ar S-oligo to have impaired production of both soluble and membrane-bound forms of Ar-- if

either or both forms are indeed synthesized by embryos and these other cell types. At this time there is no published information regarding the potential for an interaction between cell membrane-bound Ar and EGF receptors on adjacent cells.

The antisense Ar S-oligo significantly reduced mean embryo cell number and reduced the rate of blastocyst formation when compared to control embryos cultured in a random control S-oligo at 50 and 25 μM. Culturing embryos in the presence of both TGF-α and the antisense Ar S-oligo significantly improved the rate of blastocyst formation, but not mean embryo cell number. These observations indicate that TGF-α may substitute partly, but not entirely, for Ar function. The inability of TGF-α to rescue cell numbers for embryos cultured in the antisense Ar S-oligo might be due to the unique ability of Ar to localize to the nucleus and bind to DNA. Support for this hypothesis comes from the observation that nuclear localization of schwannoma-derived growth factor (SDGF), the rat homologue of Ar, is essential for its mitogenic activity (Kimura, 1993).

The capacity of added TGF-α to offset the effects of the antisense Ar S-oligo on blastocyst formation served as a specificity control for this particular S-oligo. The rescue of blastocyst development by TGF-α in this experiment suggests that the antisense Ar S-oligo may have specifically affected Ar protein function. The antisense Ar S-oligo was used at a concentration of 50 μM as a stringent test for specificity. If the effects on blastocyst formation produced by the antisense Ar S-oligo at 50 μM were entirely due to toxicity, then one would not expect TGF-α to have had any effect on blastocyst formation. However, improved blastocyst development was observed.

Recombinant Ar has recently become commercially available which prompted us to test the hypothesis that Ar added to the culture medium of preimplatation embryos might stimulate blastocyst formation and embryo cell numbers, as predicted by our previous antisense Ar studies. Indeed, preimplantation embryos cultured in Ar from both the 2-cell and 8-cell stage exhibited greater numbers of blastocysts and increased mean embryo

cell numbers compared to controls. As stated earlier, we have found that addition of TGF-α to the culture medium of preimplantation embryos stimulates primarily blastocyst formation rather than embryo cell number. Other studies have reported similar findings. For example, TGF-α has been shown to stimulate blastocoel expansion but not mean embryo cell number (Dardik and Schultz, 1991). Paria and Dey found that EGF did not increase embryo cell number when embryos were cultured in groups or singly in 50 μL drops of culture medium; EGF and TGF-α were observed to stimulate both blastocyst formation and embryo cell number when embryos were cultured singly in drops of 25 μL (Paria and Dey, 1990). Although Paria and Dey reported that EGF had no effect on blastocyst formation when embryos were cultured in groups, our studies show a clear stimulation of blastocyst formation by both TGF-α and by Ar. This apparent discrepancy may be due to how the data was collected. In our studies, we routinely monitor embryos for the first signs of cavitation and at 2 h intervals thereafter. As a result, we can be sure to record any differences in blatocyst formation between control embryos and those cultured in the growth factor. After a total of 48 h in culture, we oftentimes see equal numbers of blastocysts in each group. In Paria and Dey's study, they state that no differences in development were observed between control embryos and those cultured in EGF after 48 h in culture. It is possible that differences in blastocyst formation occurred prior to the 48 h timepoint, as we observe in our studies.

In summary, we have provided evidence that murine preimplantation embryos express the mRNA and protein for an additional EGF receptor ligand, Ar. We further propose that Ar shares with TGF-α the functions of promoting blastocyst formation and cell proliferation by initiating signal transduction cascades that serve activated EGF receptor.

However, our results suggest that Ar may be more mitogenic in preimplantation embryos than is TGF-α. We speculate that this difference may be due to the unique capacity of Ar to localize to the nucleus.

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Figure Legends

Figure 1: Indirect immunofluorescence assays using an anti-Ar IgG showing the expression of Ar protein in morulae- and blastocyst-stage preimplantation mouse embryos. **a:** Phase-contrast image of control embryos exposed to the anti-Ar IgG that was preabsorbed with the immunizing peptide. **b:** Epifluorescence image of control embryos from panel a. **c:** Epifluorscence image of morulae- and blastocyst-stage embryos exposed to the anti-Ar IgG (40 μg/mL). Note that the rhodamine signal localizes to both the cytoplasm and nucleus. **d:** Another epifluorescence image of the same embryos from panel c with a different focus emphasizing the nuclear localization in the blastocysts. The bar in panel d represents 100 μm.

Figure 2a: Ethidium bromide-stained agarose gel showing the expression of Ar mRNA by mouse blastocysts using RT-PCR and primers specific for mouse Ar. **Lane 1:** 100 base pair ladder. **Lane 2:** 434 bp RT-PCR product from 100 mouse blastocysts. **Lane 3:** PCR negative. For this control, sterile water was substituted for cDNA and the sample was then subjected to PCR. **Lane 4:** RT negative. For this control, sterile water was substituted for blastocysts and the sample was then subjected to RT-PCR.

Figure 2b: Ethidium bromide-stained agarose gel showing the results of restriction enzyme digestion of the 613 bp RT-PCR product from blastocysts. The lower primer was the same as that used for Fig. 2a, but a different upper primer was used which resulted in a bigger PCR product. **Lane 1:** Ar RT-PCR product treated with Taq1, an enzyme that should cut the Ar RT-PCR product once. Note the 613 bp product is cut into 432 bp and 181 bp restriction fragments. **Lane 2:** Ar RT-PCR product treated with EcoR1, an enzyme that should not cut the Ar RT-PCR product. Note the 613 bp product is not affected. **Lane 3:** 100 bp ladder.

Figure 3a: Effect of the antisense Ar S-oligo on embryo blastocyst formation and cell number at a concentration of 25 μM. The data represented in this graph was pooled from three separate experiments. The percentage of embryos undergoing blastocyst formation for each group is indicated by the cross-hatched bars and the numbers used to calculate this percentage are shown at the top of each bar. Embryos cultured in the antisense S-oligo exhibited a significantly reduced number of embryos undergoing blastocyst formation compared to controls (the letter "b" represents $p < .001$). The mean embryo cell number for each group is given at the top of each stippled bar (the letter "b" in this case represents $p < .05$ while the letter "c" represents $p < .001$).

Figure 3b: Effect of the antisense Ar S-oligo on embryo blastocyst formation and cell number at a concentration of 50 μM. The data represented in this graph was pooled from three separate experiments. Both "b" and "c" represent $p < .001$.

Figure 4: Effect of the antisense Ar S-oligo on embryo morphology. Blastocysts are indicated by arrows. **a:** Embryos cultured in media without S-oligos. **b:** Embryos cultured in the random, control S-oligo at 25 μM. **c:** Embryos cultured in the antisense S-oligo at 25 μM. **d:** Embryos cultured in the random, control S-oligo at 50 μM. **e:** Embryos cultured in the antisense S-oligo at 50 μM. Note that the embryos in both antisense groups are delayed at the morula stage of development. The bar in panel e represents 100 μm.

Figure 5: Effect of culturing embryos simultaneously in the presence of the antisense Ar S-oligo and TGF-α. The data represented in this graph was pooled from three separate experiments. The embryos were cultured for 48 h and scored for incidence of blastocyst formation. The antisense S-oligo was used at a concentration of 50 μM and TGF-α was

used at a concentration of 10 ng/mL. The letter "b" represents $p < .001$. The letter "c" represents $p < .001$ when compared to "b" and $p < .05$ when compared to "a".

Figure 6: Effect of TGF-α on embryo blastocyst formation and cell number. The data represented in this graph was pooled from three separate experiments. TGF-α was used at a concentration of 10 ng/mL. The letter "b" represents $p < .005$.

Figure 7: Morphology of embryos cultured simultaneously in the presence of the antisense Ar S-oligo and TGF-α. Blastocysts are indicated by arrows. **a:** Embryos cultured in the random, control S-oligo. **b:** Embryos cultured in the antisense S-oligo without TGF-α. **c:** Embryos cultured in the antisense S-oligo and TGF-α. The bar in panel c represents 100 μm.

Figure 8a: Effect of Ar on embryo blastocyst formation over time. Embryos were cultured from the 2-cell stage in 1.0, 0.1 and 0.01 ng/ml Ar. This graph represents one experiment which was repeated three times yielding similar results each time. Asterisks denote statistical differences between embryos cultured in Ar and control embryos: *$p < .05$; **$p < .025$; ***$p < .005$; ****$p < .001$.

Figure 8b: Effect of Ar on blastocyst formation. Embryos were cultured from the 2-cell stage. These results were pooled from 3 separate experiments. Asterisks denote statistical differences between embryos cultured in Ar and control embryos: *$p < .025$; **$p < .01$.

Figure 8c: Effect of Ar on mean embryo cell number. Embryos were cultured from the 2-cell stage. These results were pooled from 3 separate experiments. Asterisk denotes a statistical difference between embryos cultured in 0.01 ng/ml Ar and control embryos ($p < .02$).

Figure 9a: Effect of Ar on embryo blastocyst formation over time. Embryos were cultured from the 8-cell stage in 10 ng/ml or 1.0 ng/ml Ar. Control embryos were exposed to equivalent amounts of BSA as embryos cultured in the presence of Ar. This graph represents one experiment which was repeated (see Fig. 9b). Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $*p < .05$; $**p < .01$; $***p < .001$.

Figure 9b: Effect of Ar on embryo blastocyst formation over time. Embryos were cultured from the 8-cell stage. Control embryos were exposed to equivalent amounts of BSA as embryos cultured in the presence of Ar. This graph represents one experiment which was repeated (see Fig. 9a). Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $**p < .01$; $***p < .005$.

Figure 9c: Effect of Ar on mean embryo cell number. Embryos were cultured from the 8-cell stage. This graph was pooled from the two experiments described in Fig. 9a and Fig. 9b. Asterisks denote statistical differences between embryos cultured in Ar and the corresponding control embryos: $*p < .05$; $**p < .002$.

Figure 1 not available at time of preparation of annual report

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Figures 2a and 2b not available at time of preparation of annual report
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Effect of Antisense Amphiregulin S-oligos (25 μM) on Embryo Blastocyst Formation and Cell Number

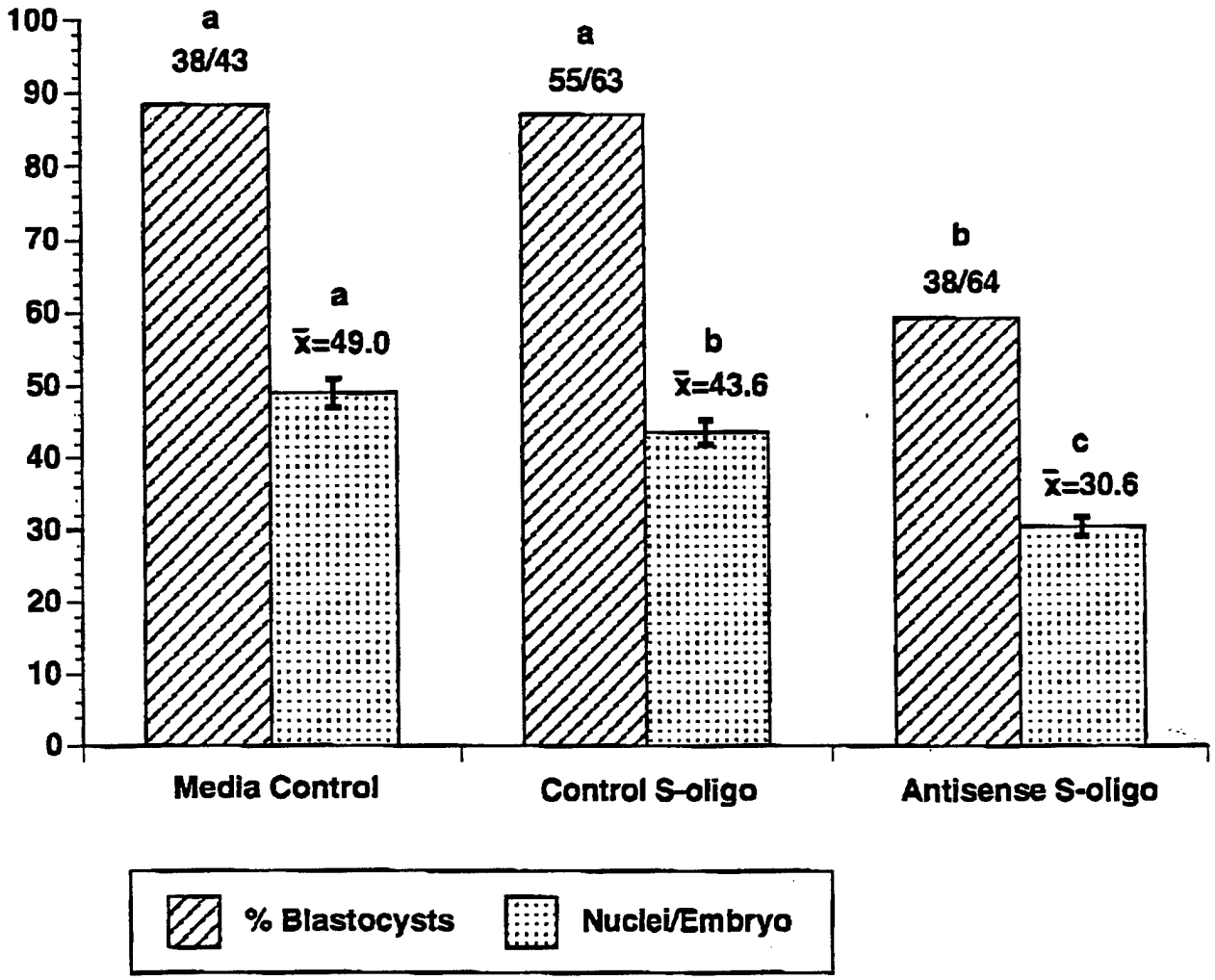

Figure 3A

Effect of Antisense Amphiregulin S-oligos (50 μM) on Embryo Blastocyst Formation and Cell Number

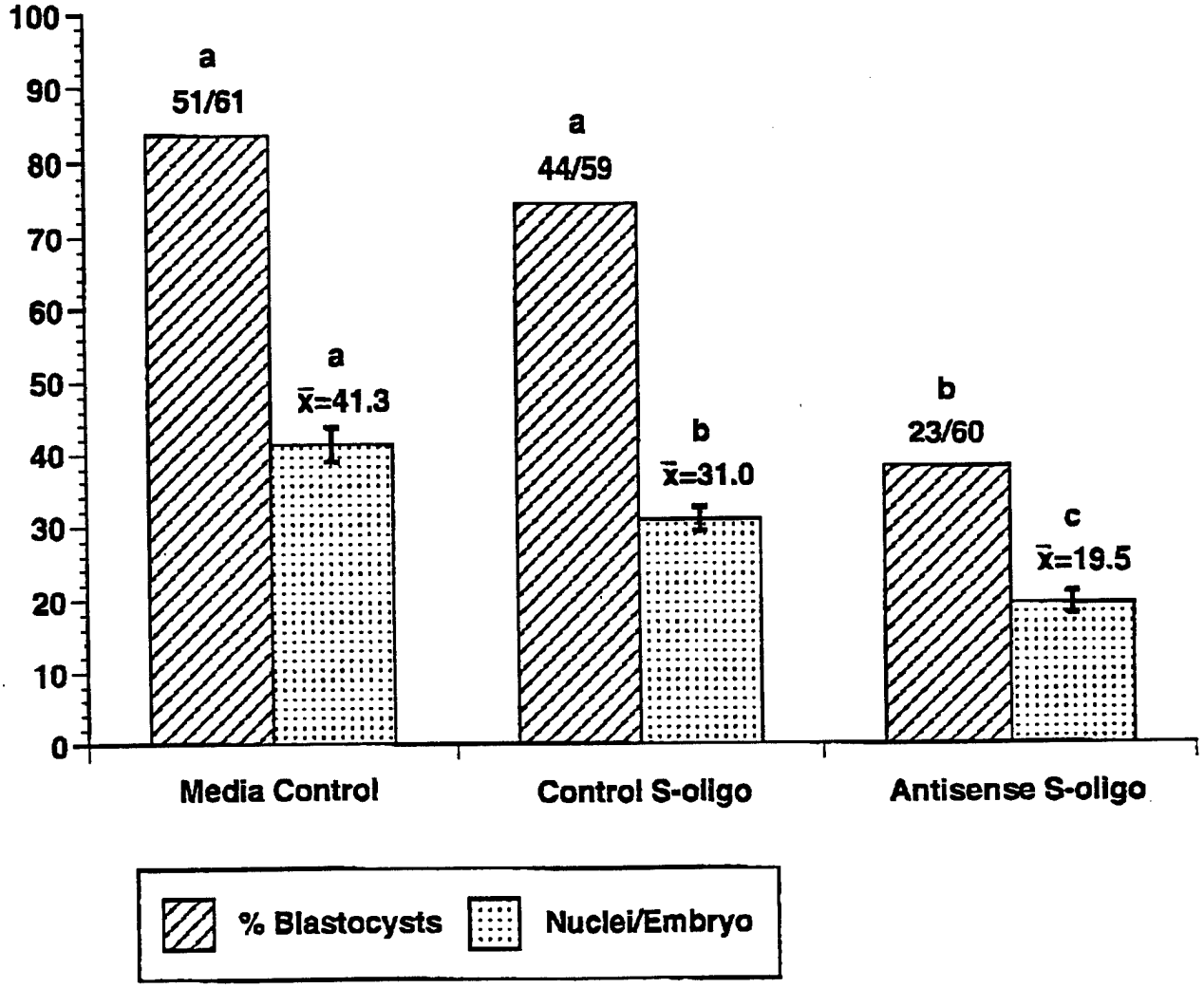

Figure 3B

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Effect of Antisense Amphiregulin S-oligos (50 μM) and TGF-α on Embryo Blastocyst Formation

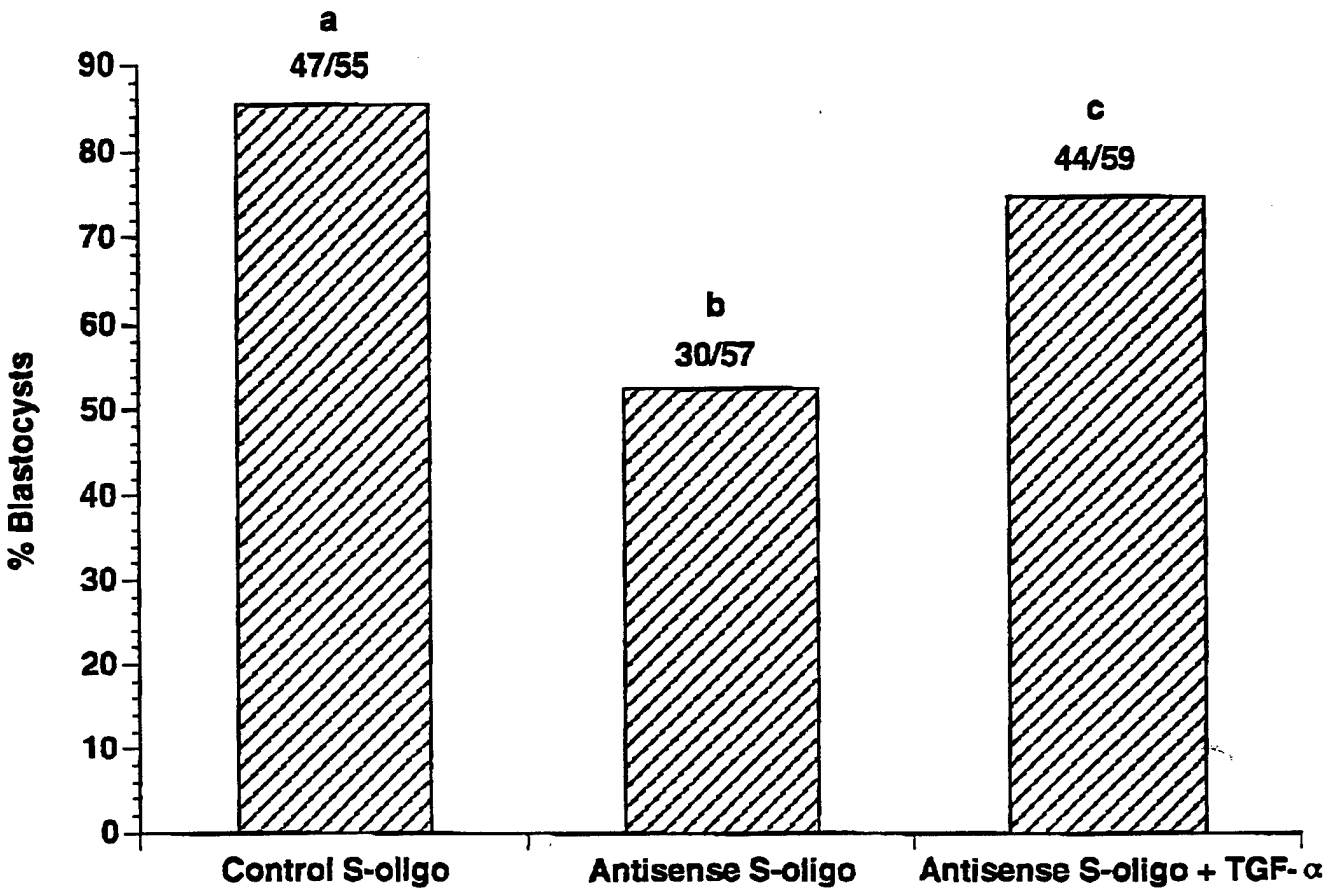

Figure 5

Effect of TGF-α (10 ng/mL) on Embryo Blastocyst Formation and Cell Number

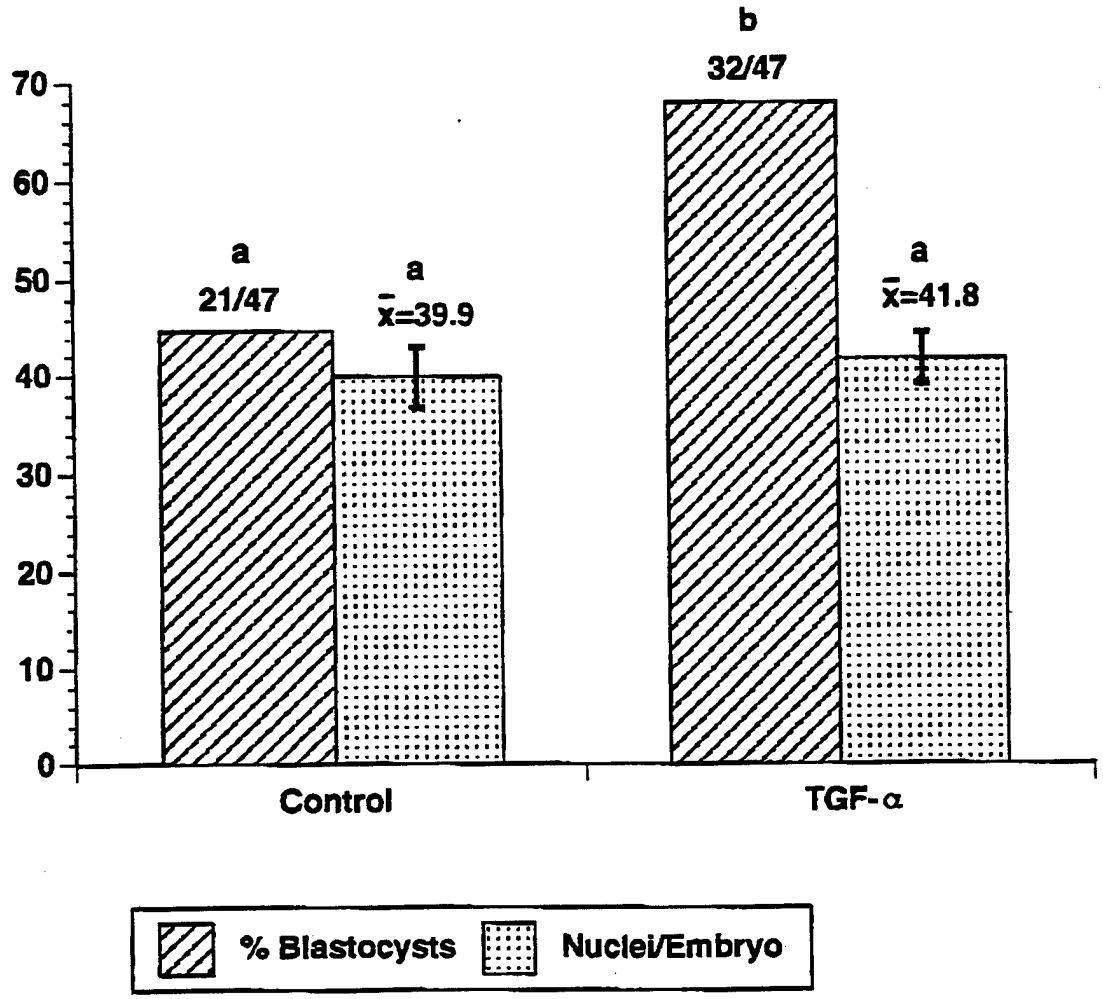

Figure 6

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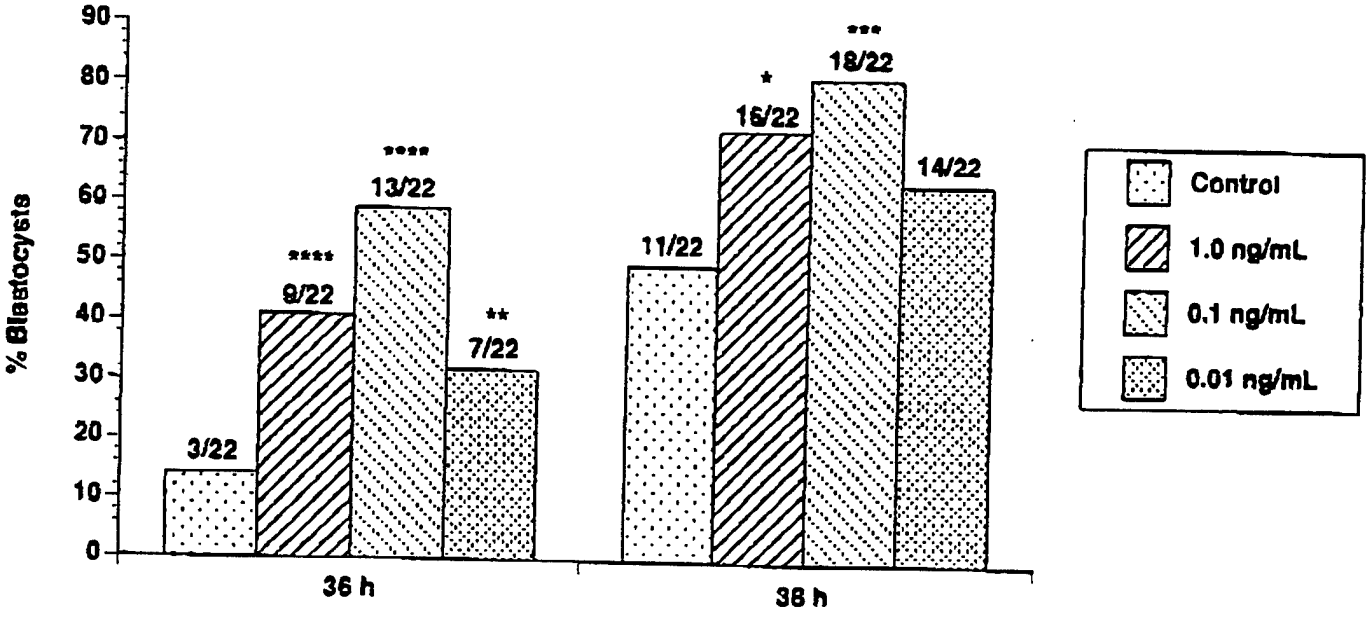
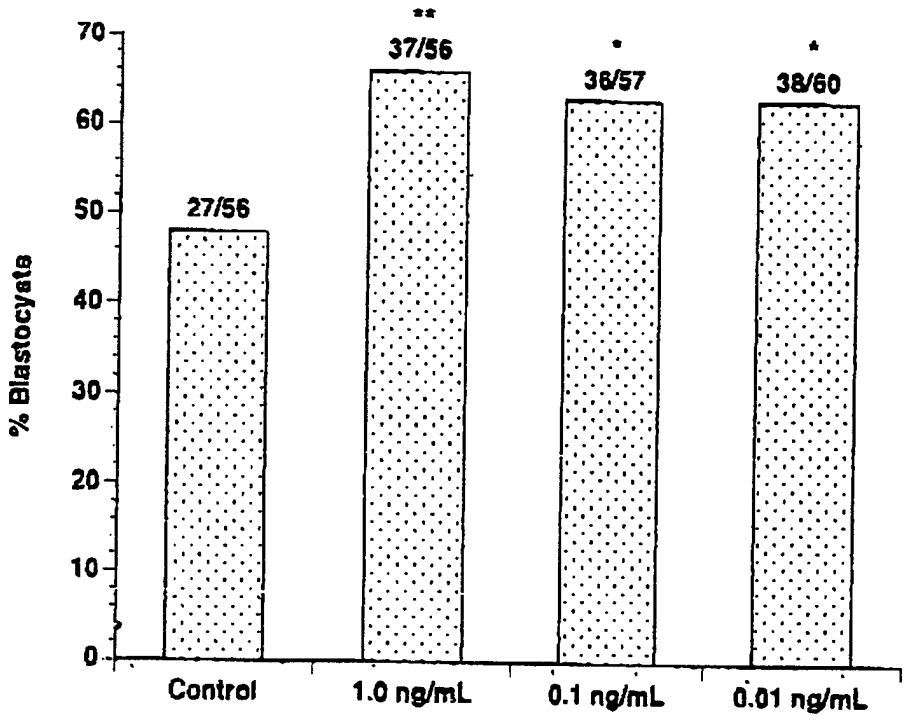


Ar Paper
Figure 8A



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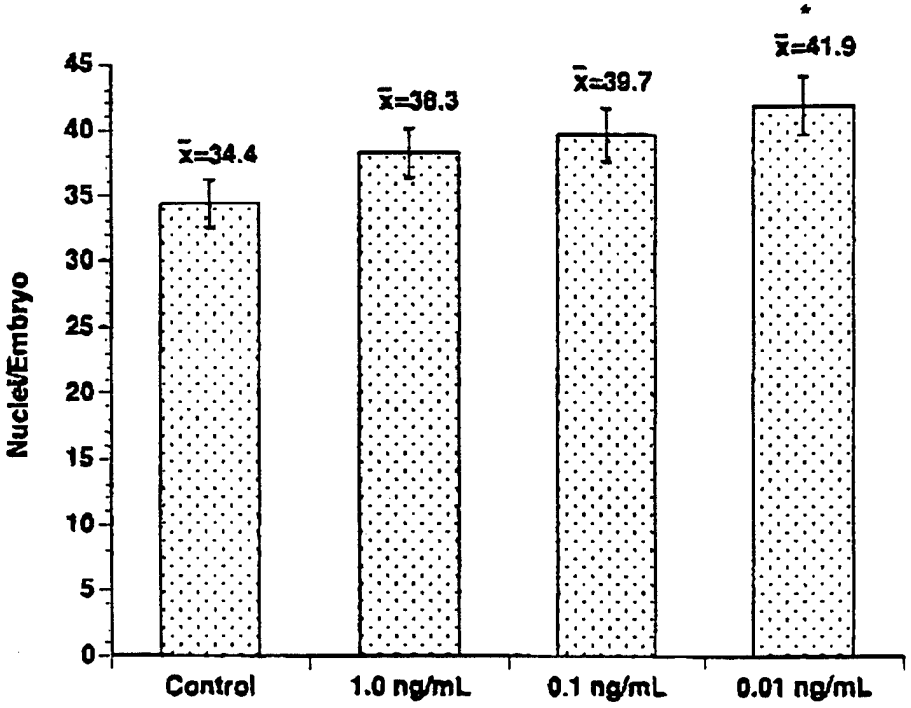

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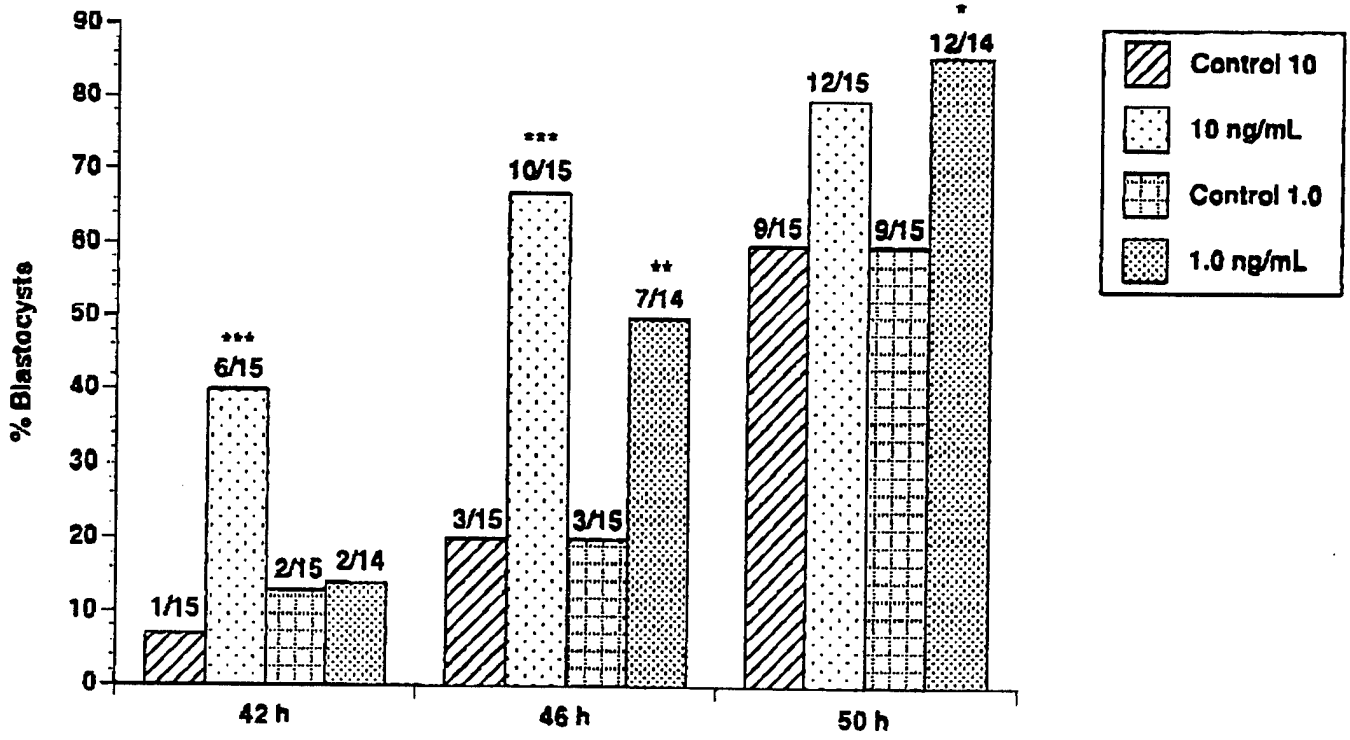

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Figure 9A

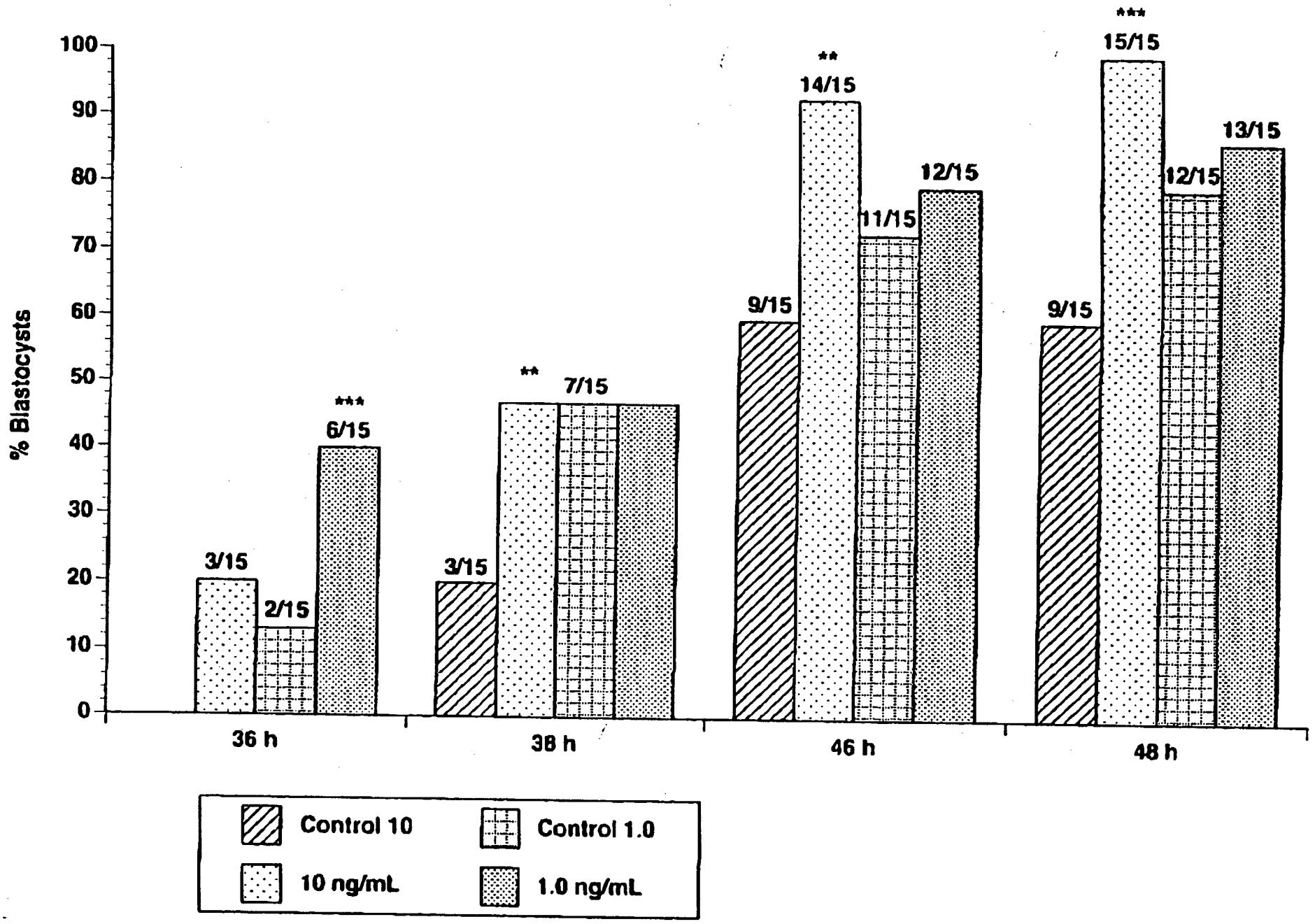

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Figure 9B

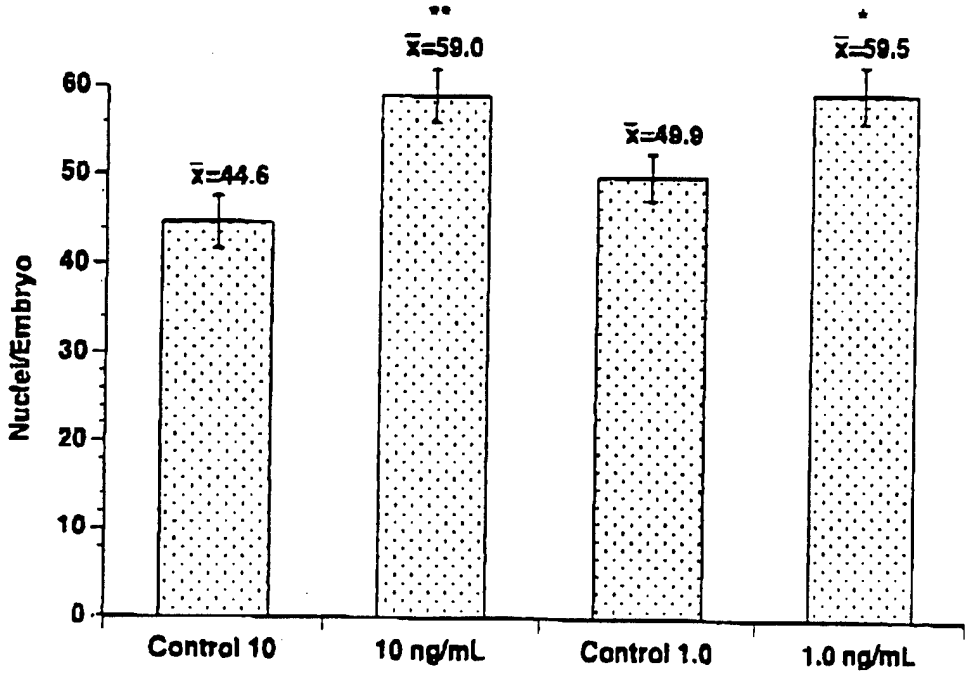

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Figure 9C